Genetic adaptation of bacteria to halogenated aliphatic compounds

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the recruitment and distribution of dehalogenase genes

Voor Marja…….

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Genetic adaptation of bacteria to halogenated aliphatic compounds:

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Voorwoord

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Chapter 1

General introduction: The evolution of degradative pathways for xenobiotic halogenated aliphatic compounds

Gerrit J. Poelarends and Dick B. Janssen

INTRODUCTION

All living organisms have a complex network of metabolic pathways for the generation of intermediates for biosynthesis and the catabolism of diverse compounds to drive cellular processes. How these pathways have evolved has been discussed for decades, and different models for pathway evolution have been postulated. These include (i) pathways have evolved in a backward direction (i.e. with the enzyme catalyzing the *N*th step of a pathway arising before the enzyme catalyzing the $N - 1$th step) (26), and (ii) pathways have evolved by a novel association of pre-existing enzymes (34). Although some of the earliest biosynthetic pathways appear to have evolved in a backward direction (11, 57), which requires special environmental conditions in which useful organic compounds and potential precursors have accumulated, it seems likely that most pathways have evolved through the recruitment and novel combination of pre-existing enzymes. A remarkable example is the Krebs cycle, which is postulated to have evolved by the combination of several pre-existing enzymes from pathways for biosynthesis of aspartate and glutamate with four additional enzymes (45).

Although the major metabolic pathways evolved millions of years ago, which makes it difficult to unravel the different events that occurred during the assembly of these pathways, pathway evolution has not ceased. At the present time, microorganisms are being challenged by the products of the chemical industry. The introduction of synthetic chemicals with structures and properties to which microorganisms have not been exposed during the course of evolution (so-called xenobiotic chemicals), have created new opportunities for microorganisms to evolve metabolic pathways in order to exploit new growth substrates or detoxify harmful compounds. Important classes of xenobiotic chemicals are the synthetic halogenated aliphatic compounds. Most synthetic halogenated aliphatics did not occur in the biosphere in significant concentrations before their industrial synthesis started some 100 years ago. They are frequently used in bulk quantities (see Table 1) and their chemical structures and properties are often distinct from those of natural organohalogens. Biodegradation of these compounds therefore usually requires the evolution of new catabolic pathways, which can be formed by acquisition of novel genes and mutations in existing genes. Thus,

microorganisms that degrade xenobiotic halogenated alipahtics are especially suited to study the natural evolution of enzyme specificity and the assembly of catabolic routes.

The remainder of this chapter will therefore describe the biochemistry and genetics of the various catabolic pathways that enable microorganisms to degrade harmful synthetic halogenated aliphatics. It will be shown that the cleavage of the carbon-halogen bond by dehalogenases is a critical step, and the evolution and distribution of these enzymes is discussed.

Table 1. Estimated annual world production in the 1980s of some of the most-produced halogenated aliphatic compounds (Chem & Eng. News 1991; 69:28-74)

Compound	World production (10^6 tons $year^{-1}$)	Product/application
1,2-Dichloroethane	23.0	Vinyl chloride, solvent, soil fumigant
Vinyl chloride	18.5	Polyvinylchloride (PVC)
Tetrachloroethylene	1.2	Dry cleaning/solvent
Trichloroethylene	1.0	Dry cleaning/solvent
Tetrachloromethane	1.0	Rubber
Trichloromethane	0.9	Intermediate in chemical synthesis
1,1,1-Trichloroethane	0.8	Solvent
Epichlorohydrin	0.7	Intermediate in chemical synthesis
Dichloromethane	0.6	Solvent
Chloromethane	0.5	Solvent
1,2-Dibromoethane	0.05[a]/0.005[b]	Gasoline additive, soil fumigant
1,3-Dichloropropene	0.005[c]	Soil fumigant

[a]The estimated world demand for 1,2-dibromoethane as a gasoline additive in 1992 (63).

[b]The estimated amount of 1,2-dibromoethane used worldwide as soil fumigant in 1992 (63).

[c]The estimated annual amount of 1,3-dichloropropene used on potato fields in the Netherlands (94).

BIODEGRADATION OF HALOGENATED ALIPHATIC COMPOUNDS

Various pure bacterial cultures capable of utilizing chlorinated or brominated aliphatics as growth substrate have been isolated, in most cases from contaminated ecosystems (Table 2) (reviewed in 58, 80). Some fascinating examples are those bacteria that can grow on the industrial bulk chemicals 1,2-dichloroethane, epichlorohydrin and dichloromethane, the pesticide γ-hexachlorocyclohexane, and 3-chloroacrylic acid, the hydrolytic degradation product of the nematocide 1,3-dichloropropene. The catabolic pathways in microorganisms that grow on these five xenobiotic compounds have been well studied (Fig. 1). These pathways are discussed below in more detail. Environmentally important halogenated aliphatic hydrocarbons that have not been shown to support growth

under aerobic conditions include chloroform, dichloroethylenes, trichloroethylene, tetrachloroethylene, 1,1-dichloroethane, 1,1,1-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropane. Furthermore, no pure bacterial cultures capable of utilizing the nematocidic soil fumigants and priority pollutants 1,2-dibromoethane and 1,3-dichloropropene as growth substrates were isolated before the work that is described in this thesis started.

Table 2. Examples of pure bacterial cultures that can aerobically utilize halogenated aliphatic compounds as growth substrates.

Compound	Organism	Reference
Halomethanes		
Methylchloride	*Hyphomicrobium* sp.	22
Dichloromethane	*Methylobacterium* spp., *Hyphomicrobium* spp., *Methylophilis* sp. DM11, *Pseudomonas* sp. DM1, *Methylorhabdus multivorans* DM13	44, 65
Haloalkanes		
1,2-Dichloroethane	*Xanthobacter autotrophicus*, *Ancylobacter aquaticus*	30, 82
1-Chlorobutane	*Rhodococcus erythropolis*	*Chapter 5*
1,6-Dichlorohexane	*Rhodococcus erythropolis*	*Chapter 5*
1-Bromooctane	*Pseudomonas* sp. ES-1, ES-2	70
Haloethenes		
Vinylchloride	*Mycobacterium aurum* L1	24
Haloethers		
2-Chloroethylvinylether	*Ancylobacter aquaticus*	83
Haloalcohols		
2-Chloroethanol	*Pseudomonas* sp., *Pseudomonas putida*	74, 75
2-Chloroallyl alcohol	*Pseudomonas* sp.	90
3-Chloroallyl alcohol	*Pseudomonas* sp.	6
2,3-Dichloro-1-propanol	*Pseudomonas* sp. OS-K-29	37
1,3-Dichloro-2-propanol	*Agrobacterium radiobacter* AD1, *Arthrobacter* sp. AD2, Coryneform bacterial strain AD3, *Corynebacterium* sp. N-1074	48, 53, 81
Haloalkanoic acids		
Chloroacetate	*Pseudomonas* sp.	33
Dichloroacetate	*Pseudomonas* sp.	17
2,2-Dichloropropionate	*Pseudomonas putida*	71
Haloalkenoic acids		
3-Chloroacrylic acid	Coryneform bacteria	23, 92
3-Chlorocrotonate	*Alcaligenes* sp. CC1	39

Microorganisms that utilize halogenated aliphatics as growth substrates generally produce specific dehalogenating enzymes that cleave the carbon-halogen bond (Fig. 1 and Table 3). Dehalogenases are often the initial enzymes of the catabolic route, and their

substrate range frequently determines the range of synthetic organohalogens that can be used as growth substrate by pure microbial cultures (58, 66). Obviously, the presence of metabolic pathways allowing the (partially) dehalogenated intermediates to be further metabolized is also essential for growth. This critical role of dehalogenating enzymes has made them an important subject for research on the biodegradation of xenobiotic halogenated compounds.

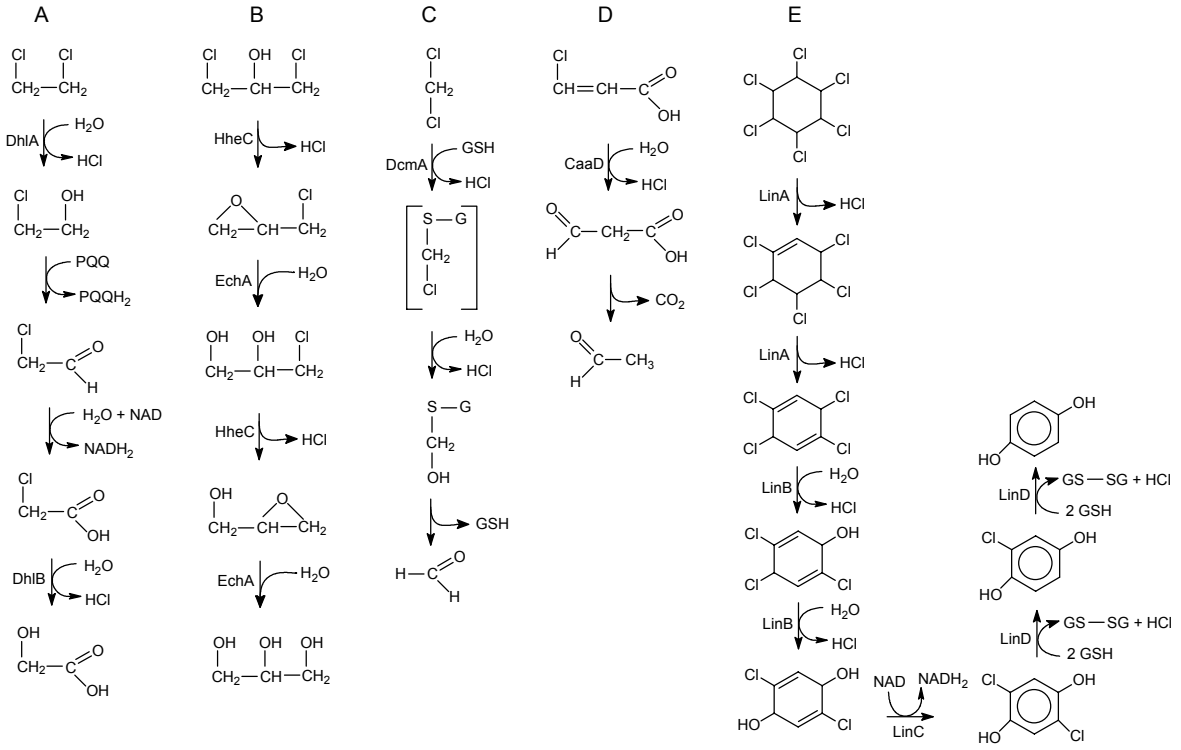

Figure 1. Pathways for degradation of halogenated aliphatic compounds. A) 1,2-dichloroethane in *Xanthobacter autotrophicus* GJ10; B) 1,3-dichloro-2-propanol in *Agrobacterium radiobacter* AD1; C) dichloromethane in *Methylobacterium* sp. strain DM4; D) 3-chloroacrylic acid in coryneform bacteria; E) γ-hexachlorocyclohexane in *Sphingomonas paucimobilis* UT26.

Several distinct mechanisms for the cleavage of carbon-halogen bonds in haloaliphatics have been described (reviewed in 10, 32). These include (i) Hydrolytic dehalogenation. In the course of hydrolytic dehalogenation reactions, catalyzed by halidohydrolases such as haloalkane dehalogenases (e.g. DhlA) and haloacid dehalogenases (e.g. DhlB) (Fig. 1A), the halogen substituent is replaced in a nucleophilic substitution reaction by a water-derived hydroxyl group. (ii) Intramolecular substitution. Halohydrin dehalogenases, such as HheC (Fig. 1B), catalyze the nucleophilic displacement of a halogen by a *vicinal* hydroxyl function in halohydrins to yield epoxides. (iii) Thiolytic dehalogenation. In dichloromethane-utilizing bacteria, glutathione *S*-transferases, such as DcmA (Fig. 1C),

catalyze the formation of an *S*-chloromethyl glutathione conjugate, with a concomitant dechlorination taking place. (iv) Dehydrohalogenation. In this type of dehalogenation, catalyzed by dehydrohalogenases such as LinA (Fig. 1E), HCl is eliminated from the molecule, leading to the formation of a double bond. (v) Reductive dehalogenation. In the course of a reductive dehalogenation, catalyzed by reductive dehalogenases such as the glutathione-dependent enzyme LinD (Fig. 1E), the halogen substituent is replaced by hydrogen. (vi) Hydration. 3-Chloroacrylic acid dehalogenases, such as CaaD (Fig. 1D), catalyze the nucleophilic addition of a water molecule on an sp^2-hybridized carbon atom of a vinylic substrate, leading to the formation of an unstable intermediate which decomposes to an aldehyde with concomitant release of halide (see *Chapter 6* of this thesis).

EXAMPLES OF CATABOLIC PATHWAYS

Utilization of 1,2-dichloroethane

Several members of the genera *Xanthobacter*, *Pseudomonas* and *Ancylobacter* that are able to utilize 1,2-dichloroethane (DCE) have been isolated from polluted sites in the Netherlands and Germany (Table 4). The best-studied DCE degrading organism is *Xanthobacter autotrophicus* GJ10 (30). This gram-negative bacterium degrades DCE through the pathway shown in Fig. 1A. In the first step, DCE is hydrolyzed to 2-chloroethanol by a constitutively produced haloalkane dehalogenase (DhlA) (31). DhlA belongs to a superfamily of hydrolytic enzymes, including acetylcholine esterase, wheat carboxy peptidase and lipases, which adopt the α/β hydrolase fold (Table 3) (12, 56). The intermediate 2-chloroethanol is oxidized to 2-chloroacetate by two inducible dehydrogenases, of which the alcohol dehydrogenase is a periplasmic enzyme. Chloroacetate is converted to glycolate by a second, constitutively produced hydrolytic dehalogenase, L-2-haloacid dehalogenase (DhlB). DhlB is a member of the large HAD (haloacid dehalogenase) superfamily of hydrolases (60) (Table 3). This HAD superfamily also comprises P-type ATPases, phosphatases, epoxide hydrolases and several other L-2-haloacid dehalogenases (3, 40). Glycolate is taken up in the central metabolic route and used for the generation of energy and cell components. A similar catabolic pathway is present in the other DCE degraders listed in Table 4.

Apart from the two hydrolytic dehalogenation steps, the rapid conversion of the toxic chloroacetaldehyde intermediate by a modified aldehyde dehydrogenase is also an essential step in DCE metabolism (76, 87, 88). The alcohol dehydrogenase is the same as the quinoprotein methanol dehydrogenases that function in methanol oxidation in gram-negative methylotrophs (29).

Table 3. Examples of dehalogenases involved in the biodegradation of haloaliphatics

Gene	Location[a]	Expression[b]	Protein	(Super)family	Pathway	Ref.
dhlA	p	c	Haloalkane dehalogenase	α/β-Hydrolase fold	1,2-Dichloroethane	31
dhaA	p	i	Haloalkane dehalogenase	α/β-Hydrolase fold	1-Chlorobutane	41
linB	c	c	Chlorohydrolase	α/β-Hydrolase fold	Hexachlorocyclohexane	49
hheA	-[c]	i	Haloalcohol dehalogenase	SDR proteins	1,3-Dichloro-2-propanol	100
hheB	-	i	Haloalcohol dehalogenase	SDR proteins	1,3-Dichloro-2-propanol	100
hheC	-	i	Haloalcohol dehalogenase	SDR proteins	1,3-Dichloro-2-propanol	u.d.
caaD1/2	-	c	3-Chloroacrylic acid dehalogenase	4-Oxalocrotonate tautomerases	1,3-Dichloropropene	u.d.
linA	c	c	Dehydrohalogenase	-	Hexachlorocyclohexane	28
linD	-	i	Reductive dehalogenase	Maleylacetoacetate isomerases	Hexachlorocyclohexane	46
dcmA	p/c	i	Dichloromethane dehalogenase	Glutathione transferases	Dichloromethane	42
dhlB	c	c	L-2-Haloacid dehalogenase	Haloacid dehalogenase (HAD)	1,2-Dichloroethane	86

[a]Abbreviations: p, plasmid; c, chromosomally encoded.

[b]Abbreviations: c, constitutive; i, inducible expression.

[c]Dashes indicate no data available.

Other abbreviations: SDR proteins, short-chain dehydrogenases/reductases; u.d., unpublished data of Janssen and coworkers.

Thus, in contrast to the other three enzymes of the catabolic route, alcohol dehydrogenase seems to be an ancient enzyme, not adapted to DCE. The gene encoding haloalkane dehalogenase (*dhlA*) is located on a large plasmid, designated pXAUI (76), and its G+C content (58%) is significantly lower than that of total DNA of a type strain of *X. autotrophicus* (65-68%). In contrast, the G+C content of the chromosomally located L-2-haloacid dehalogenase gene (67%) is close to the total G+C content. These observations suggest that of the two dehalogenase genes, only the haloalkane dehalogenase gene has recently been recruited by *X. autotrophicus*, most likely via plasmid transfer. The chloroacetaldehyde dehydrogenase gene is also located on plasmid pXAUI (76), but this gene is not closely linked to the *dhlA* gene.

These 1,2-dichloroethane-utilizing microorganisms seem to possess all the enzymes necessary to degrade the priority pollutant 1,2-dibromoethane. Surprisingly, however, 1,2-dibromoethane is toxic for these strains in the micromolar range. The absence of a functional aldehyde dehydrogenase, which results in the accumulation of the highly reactive bromoacetaldehyde, seems to be the cause of the lack of utilization of 1,2-dibromoethane for growth by 1,2-dichloroethane-utilizing bacteria (89).

Table 4. List of 1,2-dichloroethane-degrading strains carrying the *dhlA* gene

Strain	Origin	Reference
X. autotrophicus GJ10	The Netherlands, soil and sediment from different sites	30
X. autotrophicus GJ11	The Netherlands, sediment from the Rhine River	82
A. aquaticus AD20, AD25, AD27	The Netherlands, sediment from the Eems channel	82
Pseudomonas sp. RB8	Germany, industrial wastewater-treatment plant	82
Xanthobacter-like organisms: strains KDE1, KDE2, KDE3, KDE4, KDE5	Germany, soil from Braunschweig region	Dr. M. Kästner

Utilization of epichlorohydrin

The gram-negative bacterium *Agrobacterium radiobacter* AD1, isolated from freshwater sediment polluted with synthetic organohalogens, can utilize 1,3-dichloro-2-

propanol as the sole source of carbon and energy (81). The catabolic pathway for 1,3-dichloro-2-propanol via epichlorohydrin involves four steps catalyzed by only two enzymes (Fig. 1B). A haloalcohol dehalogenase (HheC) (also termed halohydrin dehalogenase or halohydrin hydrogen-halide lyase) converts the *vicinal* haloalcohols to epoxides and an epoxide hydrolase (EchA) converts the epoxides to diols. The genes encoding these two enzymes were recently cloned and sequenced (61, Van Hylckama Vlieg et al., unpublished data). Whether these genes are plasmid or chromosomally localized is not known.

A similar degradative pathway for 1,3-dichloro-2-propanol was found in the gram-positive bacterium *Corynebacterium* sp. strain N-1074 (48, 53). This organism produces two different haloalcohol dehalogenases, termed HheA and HheB (101). The corresponding genes were cloned and sequenced, and were found to be associated with the genes for two different epoxide hydrolases (101). Thus, in contrast to strain AD1, strain N-1074 seems to make use of four different enzymes to catalyze the transformation of 1,3-dichloro-2-propanol to glycerol.

Based on sequence similarities, site-directed mutagenesis experiments and X-ray crystallographic studies, it was concluded that the epoxide hydrolase of *Agrobacterium radiobacter* AD1 possesses an α/β-hydrolase fold structure with a catalytic triad similar to the one found in haloalkane dehalogenase (55, 61). Thus, EchA and DhlA share both structural and mechanistic similarities. Thusfar, three different classes of haloalcohol dehalogenases have been found, which are represented by the enzymes HheA, HheB, and HheC (see Table 3). Sequence analysis showed that these proteins share significant similarity with short-chain dehydrogenases/reductases (SDR proteins) (Van Hylckama Vlieg et al., unpublished data), which catalyze the reduction of carbonyl functions or oxidation of alcohol functions in a wide variety of substrates (35). Based on the apparent homology and the conservation of active site residues, Van Hylckama Vlieg et al. (unpublished data) proposed that haloalcohol dehalogenases and SDR proteins possess a similar catalytic mechanism. The role of the active site residues in HheC has been validated by site-directed mutagenesis (Van Hylckama Vlieg et al., unpublished data).

Utilization of dichloromethane

Several gram-negative facultative methylotrophic bacteria utilize dichloromethane (DCM) as the sole source of carbon and energy. Dichloromethane utilization begins with the dehalogenation of DCM (Fig. 1C). DCM dehalogenase, which is a member of the glutathione *S*-transferase (GST) superfamily (Table 3) (42), catalyzes a typical GST-type reaction in which glutathione attacks DCM to form a thioether intermediate which decomposes nonenzymatically to form formaldehyde, with the regeneration of glutathione (7). Formaldehyde is a central metabolite of methylotrophic growth, and is further metabolized via formate to CO_2.

The bacterial DCM dehalogenases have been shown to fall into two classes, type A and B, which differ from each other with respect to kinetic properties and amino acid sequence (5). The dehalogenases of *Methylobacterium* sp. strain DM4 (type A) and *Methylophilus* sp. strain DM11 (type B) typify the two classes. The DM4 and DM11 enzymes share 56% sequence identity (96). The DM4 enzyme has a k_{cat} of 0.6 s^{-1}, a K_m for DCM of 9 μM, and is expressed at high levels (22 to 54% of total soluble protein). The DM11 enzyme has a k_{cat} of 3.3 s^{-1}, a K_m of 59 μM, and is expressed at lower levels (9 to 27%). Gisi et al. (16) have shown that bacteria containing the DM11 enzyme quickly outcompeted strains containing the DM4 enzyme under batch conditions with an initial concentration of 10 mM DCM. In contrast, the DM4 dehalogenase was found to confer a selective advantage for growth in continuous culture in the presence of a low micromolar steady-stead level of DCM. The different properties of the DM4 and DM11 enzymes may have suited the needs of their respective hosts (16). The DM11 strain was isolated from a spill site that had been exposed to high levels of DCM for decades (67), whereas the DM4 strain was isolated from a wastewater sludge sample that had presumably been exposed to low concentrations of DCM (16).

Utilization of 3-chloroacrylic acid

Cis- and *trans*-specific 3-chloroacrylic acid dehalogenases are produced by both gram-positive and gram-negative bacteria, including *Pseudomonas cepacia* CAA1 (23) and the coryneform bacterial strains FG41 (92) and CAA2 (23), enabling these organisms to use one or both isomers of 3-chloroacrylic acid for growth (Fig. 1D). The dehalogenation product of 3-chloroacrylic acid, malonic acid semialdehyde, is converted to acetaldehyde and CO_2 by a malonate semialdehyde decarboxylase (23). The dehalogenases from strain FG41 were purified to homogeneity and the *trans*-3-chloroacrylic acid dehalogenase was found to be a 50 kDa enzyme composed of different subunits of 8.7 and 7.4 kDa, whereas the *cis*-3-chloroacrylic acid dehalogenase was an enzyme composed of two or three identical 16 kDa subunits (92). Both enzymes are produced upon induction with their respective substrates.

The 3-chloroacrylic acid dehalogenases form a fascinating class of dehalogenating enzymes since they can cleave the carbon-halogen bond in an unactivated vinylic substrate. No bacterial 3-chloroacrylic acid dehalogenase gene had been cloned before the work that is presented in this thesis started. In *Chapter 6*, we describe the gene cloning and characterization of the *trans*-3-chloroacrylic acid dehalogenase from the gram-negative bacterium *P. pavonaceae* 170. The results indicate that *trans*-3-chloroacrylic acid dehalogenases share both structural and mechanistic similarities, and a probable common ancestry, with members of the recently identified superfamily of 4-oxalocrotonate tautomerase-related enzymes (see Table 3). This superfamily also includes 5-carboxymethyl-2-hydroxymuconate isomerase, D-dopachromome tautomerase and macrophage migration inhibitory factor (47). Based on the subunit size and the conservation of putative active site

residues, determined by amino-terminal protein sequencing, c*is*-specific 3-chloroacrylic acid dehalogenase may also belong to this superfamily (*Chapter 6*).

Utilization of γ-hexachlorocyclohexane

The gram-negative bacterium *Spingomonas paucimobilis* UT26, isolated from soil that was repeatedly treated with the insecticide γ-hexachlorocyclohexane (also called lindane), can utilize γ-hexachlorocyclohexane (HCH) as the sole source of carbon and energy (27). Strain UT26 produces three dehalogenases that are essential for the degradation of HCH (Fig. 1E). The first dehalogenating enzyme of the HCH pathway, HCH dehydrochlorinase (LinA), converts HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (TCDN) by two sequential steps of dehydrochlorination. The LinA protein is encoded on the chromosome by the *linA* gene (28). LinA shows no homology to any known protein (28). The reaction catalyzed by LinA is unique and its mechanism is still unknown. The chromosomal *linB* gene encodes TCDN chlorohydrolase (LinB), the second dehalogenase of the pathway (49). This enzyme converts TCDN to 2,5-dichloro-3,5-cyclohexadiene-1,4-diol (DDOL) by two sequential hydrolytic dehalogenation steps. LinB shows significant sequence similarity to haloalkane dehalogenase (DhlA) from *X. autotrophicus* GJ10 (32, 49). The recently solved structure of LinB indeed confirmed that this enzyme also belongs to the superfamily of hydrolases with an α/β-hydrolase fold (Table 3) (44a). LinA and LinB are proposed to be periplasmic proteins (52). The intermediate DDOL is converted to 2,5-dichlorohydroquinone (DCHQ) by DDOL dehydrogenase, the product of the chromosomal *linC* gene (50). LinC shows homology to members of the short-chain alcohol dehydrogenase family (50). The third dehalogenase of the HCH pathway is encoded by the *linD* gene. The LinD protein is a glutathione-dependent dehalogenase that converts DCHQ to hydroquinone by two sequential reductive dehalogenation steps (46). LinD shares significant sequence similarity to bacterial glutathione transferases (46). Interestingly, whereas the *linA*, *linB* and *linC* genes are expressed constitutively, the *linD* gene is expressed upon induction by its substrate DCHQ, which suggests the presence of a regulatory system for LinD production.

Strain UT26 is thought to have acquired the ability to mineralize HCH in the last few decades (69, 97). However, the HCH catabolic genes are neither clustered nor plasmid localized. The G+C content of the *linA* gene (53%) is significantly lower than that of the total DNA of a type strain of *S. paucimobilis* (65%) and its codon usage is different from that of other sequenced genes of this species. This suggests that this gene has been introduced from another genus with a low G+C content (28). The G+C content of the *linB* (62.5%), *linC* (64.3%) and *linD* (60.8%) genes is close to a total G+C content of 65%. Thus, the HCH catabolic genes may have been recruited separately and from different origins. It will be interesting to find out whether these genes are flanked by insertion elements, a genetic

organization observed for various catabolic genes that are under selective pressure (84, 85, 99).

EVOLUTION OF DEHALOGENASES

As stated above, dehalogenases play important roles in the biodegradation of halogenated pollutants that were recently introduced into the environment. This suggests that enzymes must have been recently recruited to serve new functions in the biodegradation of these novel compounds. Subsequent mutations may improve the fitness of these recruited enzymes for their new roles. Thus, questions concerning the evolutionary origins and recent adaptation of dehalogenating enzymes should be addressed.

Origins of dehalogenase genes

For a long time, it has been assumed that halogenated hydrocarbons only enter the environment from anthropogenic sources. However, it is now clear that many halogenated hydrocarbons detected in the environment also originate from biogenic sources (18, 19, 20, 25). These mainly include halogenated aromatics, but also bromo- and chloromethanes are produced in large quantities by natural processes (20). It was this discovery that led to the proposal that genes from microbes degrading natural organohalogens were the origins of the dehalogenase genes found in bacteria utilizing synthetic organohalogens (32). Support for this hypothesis has not been provided yet, awaiting nucleic acid and protein sequence comparisons of dehalogenases from organisms degrading natural and those degrading synthetic organohalogens.

Organisms degrading naturally produced organohalogens are not the only potential source of dehalogenase genes. As has been pointed out by Babbitt et al. (4), the genes encoding the three components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS-3 may have evolved from a single gene locus encoding the enzymes of a β-oxidation pathway. Copley and coworkers (2) have recently suggested that genes for maleylacetoacetate isomerases were the ancestral sources of tetrachlorohydroquinone (TCHQ dehalogenase) and 2,5-dichlorohydroquinone (LinD; see Fig. 1E) reductive dehalogenase genes. TCHQ dehalogenase catalyzes the replacement of chlorine atoms on TCHQ and trichlorohydroquinone with hydrogen atoms during the biodegradation of pentachlorophenol by *Sphingomonas chlorophenolica* (100). The proposed evolutionary relationship between these reductive dehalogenases and maleylacetoacetate isomerases was based on the following observations. First, although TCHQ dehalogenase has an insignificant level of overall identity to the known maleyl-acetoacetate isomerases, enzymes that catalyze the glutathione-dependent isomerization of a *cis* double bond in maleylacetoacetate to the *trans* configuration

during the catabolism of phenylalanine and tyrosine, the active site region is highly conserved. Second, TCHQ dehalogenase catalyzes the isomerization of maleylacetone (an analogue of maleylacetoacetate) at a rate nearly comparable to that of a *bona fide* bacterial maleylacetoacetate isomerase. Since maleylacetoacetate isomerase is involved in a common and presumably ancient pathway for catabolism of tyrosine, while TCHQ dehalogenase catalyzes a more specialized reaction, the authors suggest that it is likely that TCHQ dehalogenase arose from a maleylacetoacetate isomerase, rather than that maleylacetoacetate isomerase arose from TCHQ dehalogenase. The possibility that both maleylacetoacetate isomerase and TCHQ dehalogenase arose from a common precursor with some other function is not satisfying, since it seems unlikely that an enzyme that was evolving to become a reductive dehalogenase would adventitiously also develop a high level of maleylacetoacetate isomerase activity (2). Most likely, subsequent mutations have improved the dehalogenase activity of the recruited double bond isomerase, because an additional maleylacetoacetate isomerase that has minimal TCHQ dehalogenase activity was identified in *S. chlorophenolica* (2).

Several other dehalogenases also share structural and mechanistic similarities with enzymes involved in the conversion of non-halogenated substrates, suggesting that they belong to the same superfamily (Table 3), where the members of a superfamily have a probable common evolutionary origin. However, the dehalogenases do not share high sequence identity with these known enzymes, indicating that the time of branching from a common ancestor occurred long ago. Thus, for most, if not all, dehalogenases the recent relatives (ancestral forms) from which they evolved in response to the introduction of halocarbons into the environment are unknown. Questions concerning the origins of dehalogenases therefore remain to be solved.

Adaptive mutations

It is usually difficult to identify adaptations to an enzyme that enhance its ability to dehalogenate a xenobiotic substrate because the ancestral ("primitive") form of the enzyme is not available. Pries et al. (59) have pinpointed a structural feature of haloalkane dehalogenase that may have allowed this enzyme to develop the ability to dehalogenate 1,2-dichloroethane. These authors investigated the adaptation of haloalkane dehalogenase (DhlA) to 1-chlorohexane, a compound hardly hydrolyzed by the wild type enzyme. For this, the *dhlA* gene was expressed in a strain of *Pseudomonas* that grows on *n*-hexanol and 12 independent mutants were selected that utilize 1-chlorohexane. An analysis of the corresponding mutated *dhlA* gene sequences showed that 6 mutants had the same in frame deletion of 33 bp, two mutants carried different single base substitutions, two mutants carried the same 6 bp duplication, and two mutants carried different duplications of 9 bp and 30 bp, respectively. The formation of the 33 bp-deletion can be explained by a DNA strand slippage mechanism

followed by excision and mismatch repair, whereas the formation of the short repeats seems to be due to an aspecific stuttering type of process, independent of existing repetitions (59). As expected, the six different mutant enzymes were found to have improved K_m or V_{max} values with 1-chlorohexane. All mutations occurred in a region encoding the N-terminal part of the cap domain of DhlA, and the authors concluded that this part of DhlA is involved in the evolution of activity toward xenobiotic substrates. Interestingly, the N-terminal part of the cap domain of wild-type haloalkane dehalogenase already contains two direct repeats positioned in frame: a 15 bp perfect direct repeat and a 9 bp imperfect direct repeat. The authors hypothesized that these direct repeats could be of recent evolutionary origin and were selected during the adaptation of an ancient dehalogenase to industrially produced 1,2-dichloroethane. Indeed, deletion of the two repeats in wild-type haloalkane dehalogenase resulted in complete loss of 1,2-dichloroethane hydrolyzing activity, but not in loss of activity for several brominated substrates such as the naturally produced dibromomethane (Poelarends, G.J., Dubbelhuis, P.F., and Janssen, D.B., unpublished data).

It should be noted that DhlA is probably the only dehalogenase for which there is really evidence that recent mutations have improved the fitness of this enzyme for its new role, i.e. the hydrolytic cleavage of the carbon-halogen bond in 1,2-dichloroethane. Although it has been suggested that several dehalogenases share a common ancestry with enzymes converting non-halogenated chemicals (Table 3), there is no evidence for recent adaptation of, for example, glutathione transferases or 4-oxalocrotonate tautomerases to improve or develop activity toward dichloromethane or 3-chloroacrylic acid, respectively.

ACQUISITION AND DISTRIBUTION OF DEHALOGENASE GENES

Gene transfer

Several studies on the genetics of the catabolic pathways for haloaliphatics have provided evidence that gene transfer processes played an important role in the generation of these pathways and in their (subsequent) distribution. Identical or highly similar dehalogenase genes have been found in phylogenetically different organisms and these genes are often plasmid localized and/or are associated with insertion elements (see Fig. 2). For example, Van den Wijngaard et al. (82) showed that the first metabolic step in the degradation of 1,2-dichloroethane in strains of *Xanthobacter autotrophicus* and of 2-chloroethylvinylether in *Ancylobacter aquaticus* strains is catalyzed by the same haloalkane dehalogenase DhlA (see Table 4). The corresponding *dhlA* genes are completely identical and located on a conserved 8.3 kb *Eco*RI fragment (Fig. 2A). In some strains, the *dhlA* genes were found to be localized on pXAU1-like plasmids, suggesting a role for plasmid transfer in the recent spread of the *dhlA* gene (82, Poelarends, G.J., Van der Ploeg, J.R., and Janssen, D.B., unpublished results).

In *Chapter 5* of this thesis we describe that several haloalkane-utilizing *Rhodococcus* strains isolated from widely separated geographical locations possess a highly conserved gene cluster encoding haloalkane catabolism (Fig. 2B). All analyzed strains carry this gene cluster on a plasmid, suggesting a role for these mobile elements in gene transfer. In *Methylobacterium* sp. strain DM4 and other phylogenetically distinct methylotrophs with a type A dichloromethane dehalogenase, the structural dehalogenase gene (*dcmA*) and the regulatory gene (*dcmR*) are located on a conserved DNA fragment that extends over 10 kb (Fig. 2C) (65). In some strains, this fragment is located on a 120-kb plasmid, whereas in others it is located on the chromosome (44). Interestingly, this highly conserved dichloromethane degradative gene (*dcm*) region is associated with three distinct insertion elements (Fig. 2C). These findings suggest that a transposon has been involved in the horizontal transfer of the *dcm* genes (65).

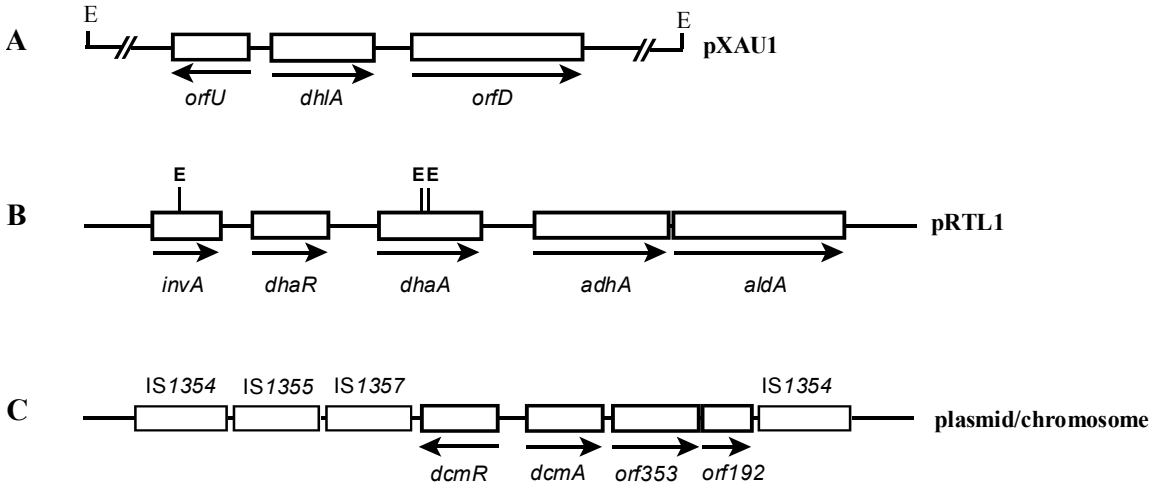

Figure 2. Genetic organization of dehalogenase regions in A) 1,2-dichloroethane-utilizing *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* strains; B) 1-chlorobutane-utilizing *Rhodococcus erythropolis* strains; and C) dichloromethane-utilizing methylotrophic bacteria with a type A dichloromethane dehalogenase.

The chlorobenzoate catabolic genes (*cbaABC*) of *Alcaligenes* sp. strain BR60 were found to be localized on a composite transposon, designated Tn*5271*, present on plasmid pBRC60 (54). This 17-kb Class I (composite) transposon is flanked by two copies of a 3.2 kb insertion element (IS*1071*), which itself is related to Class II (Tn3 family) transposons. Tn*5271* is a functional transposon as was shown by its involvement in gene transfer during the adaptation of a microbial community to several chlorinated aromatic compounds (15). This transposon was later found to be part of an even larger transposable element, designated Tn*5272* (99).

Sequential inter- and intra-molecular replicative transposition events account for the distribution of IS*1071* in multiple copies in the genomes of host bacteria (99). This combination of inter- and intra-molecular replicative transposition also suggests that IS*1071* may be found to flank other catabolic genes, forming composite transposable elements. Recently, this was found to be true. IS*1071* flanks the haloacetate dehalogenase gene *dehH2* on plasmid pUO1 in *Moraxella* sp. strain B (38, 99), the haloalkane dehalogenase gene *dhaA* on the chromosome in *P. pavonaceae* 170 (*Chapter 4*), the aniline degradative genes on plasmid pTDN1 in *Pseudomonas putida* UCC22 (14), and presumably also the *p*-sulfobenzoate degradative genes (*psbAC*) on plasmids pTSA and pPSB in *Comamonas testosteroni* strains T-2 and PSB-4, respectively (36). IS*1071* thus seems to be responsible for mobilizing these genes to the chromosome or plasmid of the respective hosts.

How an insertion element may transpose itself to a dehalogenase gene and mobilize this gene to a plasmid has been studied by Van der Ploeg et al. (88). These authors used an experimental approach to investigate the genetic adaptation of *X. autotrophicus* GJ10 to toxic concentrations of monobromoacetate (MBA). MBA can be hydrolyzed by haloacid dehalogenase (DhlB) of strain GJ10, leading to the non-toxic glycolate, but MBA concentrations above 5 mM are toxic to strain GJ10. Mutants that are able to grow in the presence of higher concentrations of MBA could easily be isolated and were found to overexpress haloacid dehalogenase. From a genetic analysis of one mutant, designated strain GJ10M50, it appeared that resistance of *X. autotrophicus* GJ10 to MBA is accompanied by the incorporation of an insertion element (IS*1247*) upstream of the *dhlB* gene. This insertion caused increased expression of haloacid dehalogenase. Another observation in their selection experiments with a strain of *X. autotrophicus* containing plasmid pPJ20 is the one-ended transposition from the chromosome to plasmid pPJ20 of IS*1247* together with *dhlB*. This also led to elevated expression of haloacid dehalogenase, which may partially be due to the increased copy number of pPJ20 compared to the chromosome. A dehalogenase gene thus may become activated by an insertion element, and the combination of a single insertion element and dehalogenase gene can transpose to another replicon.

Similar evolutionary changes have been earlier observed by Senior and coworkers (68). Their continuous culture selection experiments revealed that during a period of thousands of hours, one member of a seven-membered microbial community capable of utilizing the herbicide 2,2-dichloropropionic acid (Dalapon) acquired the ability to grow on Dalapon through the production of two dehalogenases. Apparently, cryptic dehalogenase genes in the original strain became activated during prolonged selection, most likely through the action of insertion elements (72). Several years later, Thomas et al. (77, 78) have shown that the dehalogenase gene *dehI* of *Pseudomonas putida* PP3 indeed is located on a transposable element, designated DEH.

Distribution of organisms

In some cases, the horizontal spread of dehalogenase genes is accompanied by distribution over large geographical distances. For example, gram-negative methylotrophs containing identical or nearly identical *dhlA* or *dcmA* genes were isolated from widely separated sites in Europe (see above). More strikingly, in *Chapter 5* of this thesis we describe that haloalkane-utilizing gram-positive bacteria, isolated from contaminated sites in Europe, Japan and the United States, possess identical haloalkane dehalogenase genes (*dhaA*). Such observations are difficult to interpret without invoking long-distance distribution mechanisms for microorganisms.

Despite growing concern about biological invasions and emergent diseases, not much is known about the long-distance distribution mechanisms for microorganisms. Recently, Ruiz et al. (64) have suggested that the global movement of ballast water by ships creates a long-distance dispersal mechanism for human pathogens. Similarly, the global movement of food products (for example patatoes, fruits and vegetables) by ships may have contributed to the global spread of organohalogen degrading microorganisms. Such food products are often treated with halogenated fumigants such as the food preservative 1,2-dibromoethane (1) and/or are harvested from soils treated with fumigants such as the soil disinfectant 1,3-dichloropropene (94). This may have created ecological niches were dehalogenase genes proliferate and stimulated their distribution.

REGULATION OF DEHALOGENASE EXPRESSION

A catabolic enzyme may be acquired and altered to convert a new compound, but this only yields a functional catabolic route if all other enzymes of the degradation pathway also have the required activity and are induced under the proper conditions. Acquisition of a regulatory system for gene expression is a fundamental second step in the evolution of a new catabolic route: it requires, in addition to the catabolic enzyme, a second protein molecule that can recognize and bind the compound of interest. The expression of various dehalogenases is indeed regulated by the presence of substrate, including enzymes for the conversion of halocarboxylic acids, chloroalkanes, chloroacrylic acids, chloroalcohols, 4-chlorobenzoate, and pentachlorophenol (see Table 3). Such inducible expression suggests that the catabolic pathways have well developed. Apparently, there has been enough time to evolve a regulatory system.

Only a few regulatory genes have been cloned and characterized. In the 2-haloalkanoic acids-degrading *Pseudomonas putida* PP3, the regulatory protein DehR1 responds to inducers such as 2-monochloropropionate and monochloroacetate and activates transcription of the haloalkanoic acid dehalogenase gene *dehI* (79). Transcription is mediated by an alternative

RNA polymerase containing the σ^{54} factor (78, 79). Another organohalogen sensor was discovered in dichloromethane-utilizing methylotrophs, where the negative regulator DcmR controls expression of dichloromethane dehalogenase (DcmA) in response to dihalomethanes (43, 65). In *Chapter 4*, we suggest that the product of the adjacent *dhaR* gene (Fig. 2B) regulates the plasmid-located haloalkane dehalogenase gene (*dhaA*) in various haloalkane-utilizing strains of *R. erythropolis*. The DhaR protein belongs to the TetR family of transcriptional repressor-type regulators, is a helix-turn-helix DNA binding protein, and responds to 1-chlorobutane and several other 1-halo-*n*-alkanes (9, *Chapter 4*). It is not known whether the regulatory proteins for these enzymes were designed originally to respond to natural organohalogens, and to what extent they have been modified during the development of degradative pathways for the respective halogenated xenobiotics. Thus, more insight into the importance of the fine-tuning of regulatory systems as a means of adaptation to novel substrates is needed.

The evolutionary relicts of a regulatory system can be observed in some cases. For example, in the 1,2-dichloroethane-utilizing bacterium *X. autotrophicus* GJ10, the haloalkane dehalogenase gene (*dhlA*) is expressed constitutively and addition of 1,2-dichloroethane to cells of strain GJ10 does not enhance expression (30). Surprisingly, an open reading frame (*orfU*) that is located directly upstream of the dehalogenase gene (Fig. 2A) encodes a putative regulatory protein that shares low but significant sequence similarity with members of the TetR family of repressor-type regulators (31). In addition, the promoter region of the haloalkane dehalogenase gene contains two copies of the palindromic sequence TAGGTCNNNNGACCTA, which may serve as binding sites for the putative OrfU repressor. These observations suggest that normal transcriptional regulation of the haloalkane dehalogenase gene (or its ancestral form) might have been relaxed to allow expression in the absence of an inducer. Constitutive expression is a rather primitive and wasteful approach to ensuring that the enzyme is present to participate in the degradation of 1,2-dichloroethane. However, this alteration must have been an essential step in the development of the pathway, and it may have been simpler to allow constitutive production of the dehalogenase than to modify the existing regulatory system to respond to 1,2-dichloroethane. Another example in which alteration of a regulatory protein has played an important role in the ability to utilize a new carbon source is found in *Mycobacterium* sp. strain GP1 (*Chapter 4*). This bacterium is capable of growth on the novel compound 1,2-dibromoethane by inactivation of the regulatory protein DhaR to permit constitutive production of haloalkane dehalogenase (DhaA), for which 1,2-dibromoethane is a substrate but not an inducer.

Constitutive expression of a dehalogenase thus may indicate that the enzyme is part of a recently assembled catabolic pathway that (still) lacks an (active) regulatory system. This may be illustrated by the difference in regulation of expression of haloalkanoic acid dehalogenases and dehalogenases involved in the degradation of several xenobiotic pesticides. Halogenated alkanoic acids are naturally produced, and microorganisms degrading these

compounds normally possess inducible dehalogenases (21). In contrast, halogenated pesticides (such as hexachlorocyclohexane, 1,3-dichloropropene and 1,2-dibromoethane) have been present in the environment for a short period on the evolutionary time scale, and organisms degrading these compounds constitutively produce their dehalogenases (51, *Chapters 2, 3* and *4*).

MODEL COMPOUNDS FOR STUDYING MICROBIAL ADAPTATION

The synthetic halogenated aliphatics 1,3-dichloropropene and 1,2-dibromoethane were first introduced as nematocidic soil fumigants in the 1950s, and have been used in large quantities since that time (see Table 1). They are important environmental pollutants because of their carcinogenic properties and widespread occurrence in groundwater supplies (1, 8, 73, 94, 98). These synthetic organohalogens would be expected to be highly recalcitrant to biodegradation for three reasons. First, as is obvious from their use as nematocides, they are very toxic. Second, they are unnatural compounds that have recently been introduced into the environment by human activities, and therefore microorganisms are unlikely to have complete catabolic pathways for their mineralization. Third, if degradation is initiated by a dehalogenation reaction catalyzed by an existing dehalogenase or oxidase, the lack of enzymes for the rapid conversion of the resulting halogenated alcohols or aldehydes will lead to the accumulation of toxic intermediates (93). Surprisingly, however, slow biodegradation of 1,3-dichloropropene and 1,2-dibromoethane in soils has been observed (62, 73, 91). This led to the idea that microorganisms degrading these xenobiotics must have assembled their catabolic pathways during the past few decades, possibly by the recruitment of (pre-)existing enzymes, and are thus suited to study the molecular evolution of catabolic pathways. However, until the study that is described in this thesis started, nothing was known about the genetics and biochemistry of 1,3-dichloropropene and 1,2-dibromoethane degradation because attempts to obtain pure cultures of bacteria that can degrade these compounds had been unsuccessful.

Outline of this thesis

To enquire more deeply into the nature of pathway evolution, we have studied the genetics and biochemistry of 1,3-dichloropropene and 1,2-dibromoethane degradation in two newly isolated pure bacterial cultures capable of utilizing these xenobiotics as growth substrate. In *Chapter 2* we describe the complete microbial degradation of *cis*- and *trans*-1,3-dichloropropene by the gram-negative bacterium *Pseudomonas pavonaceae* 170 (formerly *P. cichorii* 170). This organism was isolated by Van Elsas and coworkers (95) from soil that was

repeatedly treated with *cis*-1,3-dichloropropene. Strain 170 produced at least three different dehalogenating enzymes: (i) a hydrolytic haloalkane dehalogenase with activity toward both isomers of 1,3-dichloropropene; (ii) a 3-chloroacrylic acid dehalogenase specific for *cis*-3-chloroacrylic acid; and (iii) a 3-chloroacrylic acid dehalogenase specific for *trans*-3-chloroacrylic acid. The presence of these dehalogenases enables strain 170 to grow on both 1,3-dichloropropene isomers.

In *Chapter 3* we describe the isolation of a pure bacterial culture, *Mycobacterium* sp. strain GP1, that can utilize 1,2-dibromoethane as the sole source of carbon and energy. It was obtained by using a previously isolated mixed bacterial culture, capable of complete mineralization of 1,2-dibromoethane (13), as an inocculum in our further selection and adaptation experiments. This newly isolated organism metabolizes 1,2-dibromoethane via ethylene oxide by the sequential action of a haloalkane and haloalcohol dehalogenase. The latter enzyme was found to be highly active toward 2-bromoethanol and rapidly converted this toxic intermediate to ethylene oxide. This novel 1,2-dibromoethane degradative pathway employs two dehalogenases to prevent the formation of toxic brominated intermediates.

In *Chapter 4* we compare the haloalkane dehalogenase gene (*dhaA*) regions of the two nematocide degraders with that of *Rhodococcus erythropolis* NCIMB13064 (formerly *R. rhodochrous* NCIMB13064), a gram-positive haloalkane-utilizing bacterium isolated from an industrial site in the United Kingdom. From this genetic comparison, it appears that the haloalkane dehalogenase gene regions of *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1 originated from a haloalkane catabolic gene cluster similar to the one present on plasmid pRTL1 in *R. erythropolis* NCIMB13064. Most likely, distinct DNA segments containing the haloalkane dehalogenase gene were excised from such a gene cluster and were horizontally transferred to strains 170 and GP1. Furthermore, additional mutations and DNA rearrangements have occurred in the recruited DNA segments. Interestingly, both in strains 170 and GP1, a putative integrase gene was found next to the recruited DNA segment, which suggests that integration events were responsible for the acquisition of these DNA segments.

In *Chapter 5* we examine the genetic organization and genomic location of the haloalkane dehalogenase gene (*dhaA*) region in several gram-positive haloalkane-utilizing bacteria, which were isolated from geographically distinct locations. Surprisingly, all analyzed bacterial strains contained a *dhaA* gene identical to that of *R. erythropolis* NCIMB13064. Hybridization and sequence analysis of the genetic environment of the *dhaA* gene revealed high similarity to the haloalkane catabolic gene cluster found on plasmid pRTL1 in strain NCIMB13064. In all strains analyzed, the gene cluster is localized on plasmids, suggesting a role for these mobile elements in gene transfer. This globally distributed haloalkane catabolic gene cluster seems to be ancestral to the *dhaA* gene regions present in the two nematocide degraders.

In *Chapter 6* we describe the gene cloning and functional characterization of the *trans*-3-chloroacrylic acid dehalogenase (CaaD) from *P. pavonaveae* 170. CaaD is a hexameric

protein of 50 kDa that apparently consists of three α-subunits of 75 amino acid residues and three β-subunits of 70 residues. On the basis of sequence similarity, oligomeric structure, and subunit size, CaaD appears to be related to 4-oxalocrotonate tautomerase (4-OT). This tautomerase consists of six identical subunits of 62 amino acid residues and catalyzes the isomerization of 2-oxo-4-hexene-1,6-dioate to 2-oxo-3-hexene-1,6-dioate during the catabolism of catechol to metabolites in the Krebs cycle. In view of the oligomeric architecture of 4-OT, a trimer of homodimers, CaaD is postulated to function as a trimer of αβ-dimers. The sequence conservation between CaaD and 4-OT and site-directed mutagenesis experiments suggested that Pro-1 of the β-subunit and Arg-11 of the α-subunit are active site residues in CaaD. Pro-1 could act as proton acceptor/donor and Arg-11 is probably involved in carboxylate binding. Based on these findings, a novel dehalogenation mechanism is proposed for the CaaD-catalyzed reaction that does not involve the formation of a covalent enzyme-substrate intermediate. Furthermore, we propose that CaaD and 4-OT belong to the same superfamily of enzymes.

Taken together, the results described in this thesis suggest that the pathways for degradation of the xenobiotic nematocides 1,3-dichloropropene in *P. pavonaceae* 170 and 1,2-dibromoethane in *Mycobacterium* sp. strain GP1 were recently formed by the combination of (existing) dehalogenating enzymes. These apparently recently assembled metabolic pathways are associated with integrase genes, suggesting a role for DNA integrases in gene recruitment.

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Chapter 2

Degradation of 1,3-dichloropropene by *Pseudomonas cichorii* 170

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The gram-negative bacterium *Pseudomonas cichorii* 170, isolated from soil that was repeatedly treated with the nematocide 1,3-dichloropropene, could utilize low concentrations of 1,3-dichloropropene as a sole carbon and energy source. Strain 170 was also able to grow on 3-chloroallyl alcohol, 3-chloroacrylic acid, and several 1-halo-*n*-alkanes. This organism produced at least three different dehalogenases: a hydrolytic haloalkane dehalogenase specific for haloalkanes and two 3-chloroacrylic acid dehalogenases, one specific for *cis*-3-chloroacrylic acid and the other specific for *trans*-3-chloroacrylic acid. The haloalkane dehalogenase and the *trans*-3-chloroacrylic acid dehalogenase were expressed constitutively, whereas the *cis*-3-chloroacrylic acid dehalogenase was inducible. The presence of these enzymes indicates that 1,3-dichloropropene is hydrolyzed to 3-chloroallyl alcohol, which is oxidized in two steps to 3-chloroacrylic acid. The latter compound is then dehalogenated, probably forming malonic acid semialdehyde. The haloalkane dehalogenase gene, which is involved in the conversion of 1,3-dichloropropene to 3-chloroallyl alcohol, was cloned and sequenced, and this gene turned out to be identical to the previously studied *dhaA* gene of the gram-positive bacterium *Rhodococcus rhodochrous* NCIMB13064. Mutants resistant to the suicide substrate 1,2-dibromoethane lacked haloalkane dehalogenase activity and therefore could not utilize haloalkanes for growth. PCR analysis showed that these mutants had lost at least part of the *dhaA* gene.

INTRODUCTION

1,3-Dichloropropene (γ-chloroallylchloride or 1,3-dichloropropylene) is a synthetic compound that is not known to be formed naturally. The industrial production of this compound started in the 1950s as the major and active ingredient of Shell D-D and Telone II. These commercial products are mixtures of *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, and 1,2-dichloropropane and have been used worldwide in agriculture as preplant soil fumigants for control of plant-parasitic nematodes.

1,3-Dichloropropene is applied in The Netherlands to combat potato cyst nematodes on more than 30,000 ha/year. The mixture is usually applied by injection into the soil at a maximum dose of 170 kg/ha (23), which means that very large amounts (>5,000 tons/year) are used on potato fields. Although the soil is sealed by rolling, a large amount (50%) of the injected 1,3-dichloropropene evaporates into the atmosphere (23) and has a significant effect on the total air pollution caused by chlorinated hydrocarbons in The Netherlands. Fumigants such as Shell D-D and Telone II also represent an important class of carcinogenic water pollutants because their components are resistant to biological degradation and can easily permeate through soils into groundwater supplies (4, 23). The use of large amounts of these compounds in agriculture and the risk of undesirable side effects have led to several investigations into the fate and persistence of 1,3-dichloropropene and its degradation products.

Degradation of 1,3-dichloropropene in soil under laboratory and field conditions has been studied previously. Roberts and Stoydin (13) showed that both isomers of 1,3-dichloropropene were converted to the corresponding 3-chloroallyl alcohols and 3-chloroacrylic acids. Castro and Belser (3) proposed a degradation pathway for 1,3-dichloropropene and showed that the first step, chemical hydrolysis of 1,3-dichloropropene to 3-chloroallyl alcohol, does indeed take place in soil. Van Dijk (21) measured a much lower rate of disappearance of chloroallyl alcohols in sterilized soils than in nonsterilized soils, suggesting that the degradation in soil is mainly biological. These results suggest that environmental degradation of both 1,3-dichloropropene isomers is a result of microbial action, with the exception of the initial hydrolysis of 1,3-dichloropropene to 3-chloroallyl alcohol.

Bacterial degradation of 3-chloroallyl alcohol and 3-chloroacrylic acid by pure cultures has also been demonstrated (1, 6, 22), but little is known about the complete microbial degradation of 1,3-dichloropropene. The first report concerning enrichment and isolation of 1,3-dichloropropene-degrading organisms was published recently (24). In this report, Verhagen and coworkers demonstrated that repeated treatment of soils with 1,3-dichloropropene resulted in accelerated microbial degradation of this compound. Fifteen bacterial strains with 1,3-dichloropropene-degrading capacity were isolated from such adapted soils. One strain was characterized and identified as *Pseudomonas cichorii* 170, and this strain

was thought to possess a dehalogenase gene homologous to the *dhlA* gene encoding haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 (9).

Here, we describe complete microbial degradation of 1,3-dichloropropene by *P. cichorii* 170 and propose a degradation pathway. The haloalkane dehalogenase gene involved in the conversion of 1,3-dichloropropene to 3-chloroallyl alcohol was cloned and sequenced, and this gene turned out to be identical to the *dhaA* gene encoding haloalkane dehalogenase of *Rhodococcus rhodochrous* NCIMB13064 (11), which exhibits some sequence similarity to the dehalogenase gene of *X. autotrophicus* GJ10. The presence and role of the *dhaA* gene in *P. cichorii* 170 were confirmed by isolation and characterization of dehalogenase-negative mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids

The 1,3-dichloropropene-utilizing bacterium *P. cichorii* 170 was described previously by Verhagen et al. (24). Mutants of strain 170 resistant to the toxic compound 1,2-dibromoethane were isolated on MMY plates (15) containing 5 mM *cis*-3-chloroacrylic acid as a carbon source and 20 μl of 1,2-dibromoethane in the lid of each petri dish. Spontaneous mutants were observed after incubation for 2 weeks at 30°C. Four independently isolated mutants were purified and stored on Luria-Bertani (LB) medium (15). A representative mutant, strain 170M4, was chosen for further study.

Escherichia coli BL21(DE3), a strain expressing the RNA polymerase of bacteriophage T7 (19), and the vector pGEF+ (12) were used for the cloning experiments and for expression of the recombinant enzyme. *E. coli* JM101 (Promega) was used for isolation of single-stranded DNA from pGEF+ derivatives.

Media and growth conditions

Cells of strains 170 and 170M4 were grown aerobically at 30°C in MMY (15) or LB medium (15). When required, Difco agar (15 g/liter) was added to the medium. To prevent evaporation of volatile substrates, cultivation was carried out in closed flasks filled to one-fifth of their volume with medium.

E. coli strains were grown at 30°C in LB medium with rotary shaking or on solid LB medium (15). Ampicillin (100 μg/ml) was used for detection of recombinant plasmids. LBi medium, which was used for pH indicator plates, was solid LB medium supplemented with 80 mg of bromothymol blue (Merck) per liter and adjusted to pH 8.0.

Gas chromatography

Amounts of 1,3-dichloropropene and 3-chloroallyl alcohol were determined by capillary gas chromatography. Samples (1 ml) were extracted with 1 ml of diethyl ether containing 0.05 mM 1-bromohexane as an internal standard. Extracts were analyzed by split injection of 2- or 4-μl samples into a type HP-5 column (model HP 19091J-413; Hewlett-Packard) by using nitrogen as the carrier gas. The column was installed in a model 6890 gas chromatograph (Hewlett Packard) equipped with a flame ionization detector. The oven was temperature programmed as follows: 3 min (isothermal) at 40°C, followed by an increase at a rate of 10°C/min to 90°C and then an increase at a rate of 30°C/min to 140°C for 1,3-dichloropropene; and 3 min (isothermal) at 60°C, followed by an increase at a rate of 10°C/min to 180°C for 3-chloroallyl alcohol. The different isomers of 1,3-dichloropropene and 3-chloroallyl alcohol were clearly separated from each other. Typical elution times for *cis*-1,3-dichloropropene, *trans*-1,3-dichloropropene, *cis*-3-chloroallyl alcohol and *trans*-3-chloroallyl alcohol were 5.4, 4.9, 9.3, and 10.3 min, respectively.

Preparation of crude extracts

Cells of strains 170 and 170M4 were harvested in the exponential growth phase by centrifugation, washed with 1 volume of TEMAG buffer (10 mM Tris-sulfate [pH 8.2], 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02 % sodium azide, and 10% glycerol), and disrupted in an appropriate amount of this buffer by sonication. A crude extract was obtained by centrifugation (30 min at 50,000 rpm in a type 70 Ti rotor [Beckman]).

The recombinant dehalogenase was expressed in *E. coli* BL21(DE3) and a crude extract was prepared as described previously (17).

Enzyme purification

For isolation and purification of the haloalkane dehalogenase of strain 170, cells were grown in LB medium or MMY-1% citrate. The cells were cultivated at 30°C until the early stationary growth phase. The cells were harvested by centrifugation, washed with 1 volume of TEMAG buffer, and disrupted in an appropriate ammount of this buffer by sonication. Unbroken cells and debris were removed by centrifugation for 1 h at 50,000 rpm in a type 70 Ti rotor (Beckman). The crude extract was applied to a DEAE-cellulose column which was equilibrated with TEMAG buffer. The column was washed with 1 column volume of TEMAG buffer and the proteins were eluted with a linear gradient of 0 to 1 M ammonium sulfate in TEMAG buffer. Fractions that showed dehalogenase activity with 1,2-dibromoethane were pooled and dialyzed overnight against PEMAG buffer (5 mM potassium phosphate [pH 6.5], 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium-azide, 10% glycerol). The dialysate was loaded onto a hydroxylapatite column which was equilibrated with PEMAG buffer. The column was washed with 1 column volume of PEMAG buffer, and the enzyme was eluted

with a linear gradient of 0 to 100 mM potassium phosphate in PEMAG buffer. Fractions with the highest haloalkane dehalogenase activity were pooled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assays

Haloalkane and 3-chloroacrylic acid dehalogenase activities were measured by incubating an appropriate amount of enzyme or cell extract with 3 ml of 5 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (2, 10). All dehalogenase activities are expressed as units per milligram; 1 U was defined as 1 μmol of halide produced per min per mg of protein. Most enzyme assays were carried out twice, and the difference in specific activity was less than 10%.

Protein concentrations were estimated with Coomassie brillant blue by using bovine serum albumin as the standard.

Biochemical characterization

The molecular masses of denatured dehalogenases were determined by SDS-PAGE on gels containing 12.5% polyacrylamide. Phosphorylase *b* (molecular mass, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), all obtained from Pharmacia, were used as reference proteins. The gels were stained with Coomassie brilliant blue.

To determine the amino-terminal amino acid sequence of the purified haloalkane dehalogenase (DhaA), 0.5 μg of protein was electrophoresed on a 12.5% polyacrylamide SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by electroblotting, and stained with Coomassie brilliant blue. After the gel was washed with distilled water, the DhaA band was excised and immediately subjected to automated Edman degradation (Eurosequence BV, Groningen, The Netherlands).

DNA isolation

To isolate total DNA, cells of strains 170 and 170M4 were grown in 50 ml of LB medium at 30°C until the early stationary growth phase. Ampicillin (200 μg/ml) and lysozyme (100 μg/ml) were added, and the culture was incubated at 30°C for another 1 h. Cells were harvested by centrifugation and resuspended in 10 ml of 10 mM Tris-buffer (pH 8.5) containing 1 mM EDTA and 50 mM NaCl. Then 10 mg of lysozyme was added immediately, and the mixture was incubated at 37°C for 2 h. After 1.6 ml of 10 % SDS was added, the mixture was incubated at 65°C until lysis was complete. Finally, 1.2 ml of 3 M sodium acetate (pH 7.0) was added, and the mixture was incubated at 65°C for another 2 h. The preparation was extracted twice with an equal volume of phenol, then with phenol-chloroform (1/1, vol/vol), and finally with chloroform-isoamyl alcohol (24/1, vol/vol). Total

DNA was precipitated by adding 2 volumes of cold ethanol (96%) and was collected with a glass rod. After washing with 70% ethanol, the DNA was resuspended in 1 ml of 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA.

Cloning of the *dhaA* gene

The general procedures used for cloning and DNA manipulation were essentially the procedures described previously (15). To clone *dhaA*, total DNA from strain 170 was directly used for PCR amplification by the standard protocol described by Innis and Gelfand (7). Total DNA and synthetic oligonucleotide primers were each used at a concentration of 100 ng per 100 μl of total PCR mixture. The reaction was performed with GoldStar DNA polymerase by using denaturation, annealing, and extension temperatures of 94, 58, and 72°C, respectively. The DNA oligonucleotides used as primers were designed on the basis of the N- and C-terminal DNA sequences of the *dhaA* gene of *R. rhodochrous* NCIMB13064 (GSDB accession no. L49435) and had the following nucleotide sequences: 5'-AA-AATCGCC**ATG**GCAGAAATCGGTA-3' (the start codon is in boldface type, and the *Nco*I site is underlined) and 5'-TGGACATCGGACCATGGCGTGAAC**C**-3' (the C of the stopcodon is in boldface type, and the *Nco*I site is underlined). After 30 cycles of amplification, formation of a DNA product was checked by agarose gel electrophoresis (15). The PCR product was purified with a QIAquick PCR purification kit (Qiagen), digested with *Nco*I, and ligated into the *Nco*I site of the T7 expression vector pGEF+. The ligation mixture was used to transform electrocompetent cells of *E. coli* BL21(DE3) by electroporation. Transformants were plated onto LBi medium plates containing 100 μg of ampicillin per ml. Resistant colonies were screened for the presence of dehalogenase activity as described previously (17). Plasmid DNA was isolated from a colony showing dehalogenase activity and was checked by restriction analysis. The recombinant plasmid pGEF(*dhaA*) was transformed into *E. coli* JM101 for isolation of single-stranded DNA (15), which was used as the template for DNA sequencing of the inserted gene by the dideoxy chain termination method of Sanger et al. (16).

Chemicals and enzymes

Restriction enzymes, T4 DNA ligase, and molecular weight marker X were obtained from Boehringer (Mannheim, Germany). GoldStar DNA polymerase was purchased from Eurogentec (Seraing, Belgium). DEAE-cellulose was obtained from Whatman Ltd., Kent, England; and hydroxylapatite was obtained from Bio-Rad Labaratories, Richmond, Calif. All halogenated compounds, including the separate isomers of 1,3-dichloropropene, 3-chloroallyl alcohol, and 3-chloroacrylic acid, were supplied by Janssen Chimica (Beerse, Belgium) and were at least 97% pure according to the manufacturer. The DNA oligonucleotides used as primers were synthesized by Eurosequence BV.

RESULTS

Characterization of *P. cichorii* 170

The 1,3-dichloropropene-degrading organism was identified by using the BIOLOG identification system as *P. cichorii* 170 (24). Strain 170 was able to utilize the following organic compounds as growth substrates: citrate, glucose, ethanol, 1-propanol, 1-butanol, 1-pentanol, and crotonic acid. No growth occurred with methanol, allyl alcohol, acrylic acid, ethylene glycol, or toluene.

Utilization of halogenated compounds

Growth inhibition experiments performed under standard conditions showed that when *cis*- or *trans*-1,3-dichloropropene was added at a concentration of more than 0.75 mmol/liter, it was very toxic for strain 170 and completely inhibited growth on citrate. This inhibition was caused by the toxic effects of 1,3-dichloropropene itself, because the corresponding 3-chloroallyl alcohols were not toxic and could even serve as growth substrates at concentrations up to 5 mM. However, strain 170 could efficiently utilize *cis*- and *trans*-1,3-dichloropropene when each of them was added at an amount of 0.075 mmol to 3-liter flasks with a high air/medium ratio (Fig. 1). Growth resulted in disappearance of the substrate and simultaneous formation of biomass and inorganic chloride. The final chloride concentration exceeded the low initial liquid phase concentration of dichloropropenes by more than twofold since the chloroalkenes were distributed in the gas and liquid phases. Both c*is*- and *trans*-3-chloroallyl alcohol transiently accumulated in the medium, indicating that they are the intermediates formed during the conversion of *cis*- and *trans*-1,3-dichloropropenes, respectively.

We determined whether strain 170 could utilize halogenated compounds that are structurally related to 1,3-dichloropropene and its possible degradation products. Growth tests were done under standard conditions in liquid medium supplemented with different carbon sources at a concentration of 2 mM (Table 1). *P. cichorii* 170 was capable of growth with 1,3-dichloropropane and with 1-halo-*n*-alkanes containing up to at least 10 carbon atoms. The environmentally important compounds 1,2-dichloroethane, 1,2-dibromoethane, 1,2-dichloropropane, and 1,2,3-trichloropropane were not growth substrates for strain 170. The lack of growth with these compounds was probably not due to toxicity, as observed with 1,3-dichloropropene, since these compounds lack the reactive allylic halogen, which can lead to alkylation of nucleophilic groups. Strain 170 was also able to grow on *cis*- or *trans*-3-chloroallyl alcohol and *cis*- or *trans*-3-chloroacrylic acid, which are possible degradation products of the corresponding 1,3-dichloropropenes. No growth was observed with other chloroallyl alcohols or halogenated acids.

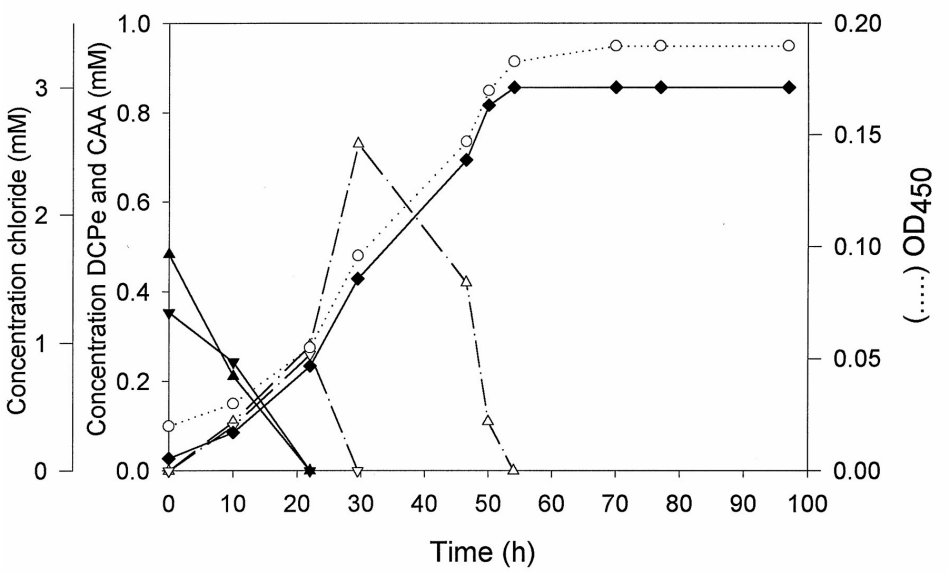

Figure 1. Growth of strain 170 on a mixture of *cis*- and *trans*-1,3-dichloropropene. A 0.15-mmol portion of 1,3-dichloropropene (ratio of *cis*-1,3-dichloropropene to *trans*-1,3-dichloropropene, 1/1 [mol/mol]) was added to 100 ml of MMY in a 3 liter flask, which resulted in a low initial liquid phase concentration. Symbols: ▲, *cis*-1,3-dichloropropene concentration; ▼, *trans*-1,3-dichloropropene concentration; Δ, *cis*-3-chloroallyl alcohol concentration; ∇, *trans*-3-chloroallyl alcohol concentration; O, optical density at 450 nm (OD_{450}); ♦, chloride concentration. Abbreviations: CAA, 3-chloroallyl alcohol; DCPe, 1,3-dichloropropene.

Metabolism of 1,3-dichloropropene

Activities of enzymes that may be involved in 1,3-dichloropropene metabolism were tested with crude extracts prepared from cells grown on citrate, 1-chlorobutane, *cis*-3-chloroacrylic acid, and *trans*-3-chloroacrylic acid (Table 2). It appeared that both isomers of 1,3-dichloropropene were converted to the corresponding 3-chloroallyl alcohols, which indicates that dehalogenation of 1,3-dichloropropene is a hydrolytic reaction in this organism. The constitutively expressed haloalkane dehalogenase had a broad substrate range (Table 3) and did not require any cofactors or metal ions for activity. The highest level of dehalogenase activity was observed with 1,2-dibromoethane, while no activity was observed with the analog 1,2-dichloroethane.

Conversion of *cis*- and *trans*-3-chloroallyl alcohol is likely to proceed via 3-chloroacrolein to the corresponding 3-chloroacrylic acid isomers (3, 13). The extracts of cells grown on different substrates did not show chloride release upon incubation with *cis*- or *trans*-3-chloroallyl alcohol (Table 2), indicating that direct dechlorination of the 3-chloroallyl alcohol isomers indeed does not occur.

The initial step in 3-chloroacrylic acid metabolism in strain 170 was studied by incubating crude extracts separately with the 3-chloroacrylic acid isomers. The extracts of cells grown on different substrates showed chloride formation when *trans*-3-chloroacrylic

acid was added. Dechlorination of *cis*-3-chloroacrylic acid was observed only with crude extracts prepared from cells grown on *cis*-3-chloroacrylic acid. These results indicate that two different enzymes are involved in the dechlorination of the 3-chloroacrylic acid isomers, a *trans*-specific dehalogenase that is constitutively expressed and an inducible *cis*-specific dehalogenase.

Table 1. Utilization of halogenated compounds by *P. cichorii* 170

Compound	Utilization	Compound	Utilization
Halogenated alkanes		**Halogenated alcohols**	
1,2-Dichloroethane	-[a]	2-Chloroallyl alcohol	-
1,2-Dibromoethane	-	*cis*-3-Chloroallyl alcohol	+
1-Chloropropane	+	*trans*-3-Chloroallyl alcohol	+
1-Bromopropane	+	3,3-Dichloroallyl alcohol	-
2-Chloropropane	-	2,3,3-Trichloroallyl alcohol	-
1,2-Dichloropropane	-		
1,2-Dibromopropane	-	**Halogenated acids**	
1,3-Dichloropropane	+	Bromoacetic acid	-
1,3-Dibromopropane	-	Chloroacetic acid	-
1-Bromo-3-chloropropane	-	2-Chloropropionic acid	-
1,2,3-Trichloropropane	-	3-Chloropropionic acid	-
1,2,3-Tribromopropane	-	3-Chlorocrotonic acid	-
1-Chlorobutane	+	*cis*-3-Chloroacrylic acid	+
1-Bromobutane	+	*trans*-3-Chloroacrylic acid	+
1-Chloropentane	+		
1-Bromopentane	+		
1-Chlorohexane	+		
1-Bromohexane	+		
1-Chlorooctane	-		
1-Chlorononane	-		
1-Bromononane	+		
1-Bromodecane	+		

[a]-, no growth; +, visible growth after one week of cultivation in liquid MMY at room temperature. In all cases, growth was accompanied by halide release. Each carbon source was added at a concentration of 2 mM.

Purification of the haloalkane dehalogenase

The haloalkane dehalogenase of strain 170 was purified 16-fold, indicating that this dehalogenase was present at a concentration equivalent to 6 to 7% of the total soluble cellular protein. After SDS-PAGE, only one protein band at approximately 33 kDa was observed (Fig. 2). The purified enzyme could be stored in TEMAG buffer at 4°C with no loss of activity. During purification, similar increases in specific activity were observed for *cis*- and *trans*-1,3-

dichloropropene dehalogenase activities, indicating that both 1,3-dichloropropene isomers were converted by the same enzyme (Table 4). The purified haloalkane dehalogenase did not catalyze conversion of *cis*- or *trans*-3-chloroacrylic acid, indicating that other dehalogenases with activities for these compounds must be present in strain 170.

Table 2. Dehalogenase activities in crude extracts prepared from cells grown on different carbon sources[a]

Enzyme substrate	Dehalogenase sp act (mU/mg of protein) with the following carbon sources:			
	Citrate	1-Chloro-butane	*Cis*-3-Chloro-acrylic acid	*Trans*-3-Chloro-acrylic acid
cis-1,3-Dichloropropene	440	280	280	256
trans-1,3-Dichloropropene	238	203	167	147
cis-3-Chloroallyl alcohol	<10	<10	<10	<10
trans-3-Chloroallyl alcohol	<10	<10	<10	<10
cis-3-Chloroacrylic acid	<10	<10	161	<10
trans-3-Chloroacrylic acid	230	63	134	177

[a]Specific activities with various substrates (5 mM) were determined with extracts prepared from cells grown on different carbon sources.

Table 3. Dehalogenating activities of a crude extract prepared from citrate-grown cells with various substrates

Substrate	Sp act (mU/mg)[a]	Substrate	Sp act (mU/mg)[a]
1,2-Dichloroethane	<10	1-Bromo-3-chloropropane	233
1,2-Dibromoethane	696	1,2,3-Trichloropropane	10
1-Chloropropane	48	1-Chlorobutane	79
1-Bromopropane	57	1-Bromobutane	44
1,2-Dichloropropane	<10	1-Chlorohexane[b]	88
1,2-Dibromopropane	189	1-Chlorononane[b]	28
1,3-Dichloropropane	119	1-Bromononane[b]	42
1,3-Dibromopropane	154	1-Bromodecane[b]	38

[a]Halide production at 30°C with different substrates (5 mM) was determined at pH 8.2.
[b]Assays were performed with substrate at a concentration of 3 mM.

The amino-terminal amino acid sequence of the purified protein of the gram-negative organism *P. cichorii* was determined to be M-S-E-I-G-T-G-F-P-F-D-P-H-Y-V-E-V, which is identical to the amino-terminal sequences of the dehalogenases of the gram-positive strains *R. rhodochrous* NCIMB13064 (11), *Rhodococcus erythropolis* Y2 (14), and *Arthrobacter* sp. strain HA1 (18).

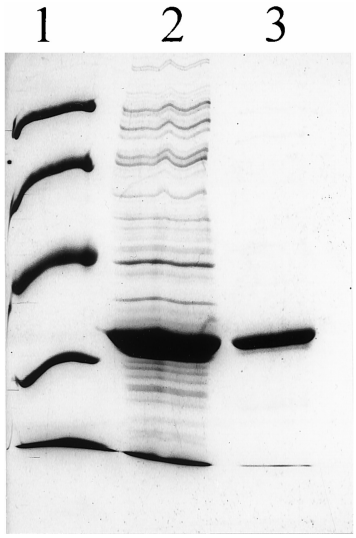

Figure 2. SDS-PAGE of crude extract of *E. coli* BL21(DE3)/pGEF(*dhaA*) (lane 2) and purified haloalkane dehalogenase from *P. cichorii* (lane 3). Lane 1 contained protein markers with molecular masses of 94, 67, 43, 30, and 20 kDa.

Cloning of the haloalkane dehalogenase gene *dhaA*

Earlier studies (24) suggested that a plasmid-located *dhlA*-like gene may be involved in 1,3-dichloropropene degradation. To determine whether a haloalkane dehalogenase gene that was homologous to the *dhlA* gene of *X. autotrophicus* GJ10 (9) was present in strain 170, Southern hybridizations were performed with the *Xanthobacter* gene as a probe. These hybridizations with total DNA of strain 170 were done under nonstringent conditions, but no positive signal was detected.

The biochemical characteristics and amino-terminal sequence of the haloalkane dehalogenase that we purified from *P. cichorii* 170 suggested that the enzyme closely resembled the haloalkane dehalogenases present in a number of gram-positive strains (5, 8, 14, 18, 25). The DNA sequence of the haloalkane dehalogenase gene of one of these gram-positive strains, *R. rhodochrous* NCIMB13064, has been published recently (11). To determine whether the haloalkane dehalogenase gene of strain 170 was identical to the *dhaA* gene of *R. rhodochrous*, the putative *dhaA* gene of strain 170 was amplified by PCR with primers based on the N- and C-terminal dehalogenase sequences of *R. rhodochrous*. Electrophoresis on an agarose gel revealed that when total DNA of strain 170 was the template, a 0.9-kb DNA product was formed by PCR. The PCR fragment was purified and cloned in the expression vector pGEF+, which resulted in production of an active dehalogenase in *E. coli* BL21(DE3). SDS-PAGE of the purified haloalkane dehalogenase of strain 170 and the *dhaA* gene product overexpressed in *E. coli* BL21(DE3) showed that the enzymes had the same electrophoretic mobility and the same molecular mass, 33 kDa (Fig. 2). The ratio of *trans*-1,3-dichloropropene dehalogenase activity to *cis*-1,3-dichloropropene dehalogenase activity was 0.5 for the purified dehalogenase and also for the overexpressed

dhaA gene product in *E. coli* (Table 4). Thus, the cloned gene indeed encodes the purified dehalogenase of strain 170. DNA sequencing of the cloned PCR-amplified dehalogenase gene revealed a sequence identical to the sequence of the *dhaA* gene of *R. rhodochrous*. Thus, the haloalkane dehalogenase gene of the gram-negative organism *P. cichorii* 170 is identical to the haloalkane dehalogenase gene of the gram-positive organism *R. rhodochrous* NCIMB 13064.

Table 4. Dehalogenating activities of crude extracts and purified dehalogenase

Enzyme substrate	Dehalogenase sp act (mU/mg of protein)[a]		
	Cell extract of strain 170	Cell extract of BL21(DE3)/pGEF(*dhaA*)	Purified dehalogenase
cis-1,3-Dichloropropene	440	4,204	7,150
trans-1,3-Dichloropropene	238	2,140	3,549
1-Chloropropane	48	449	744
1-Chlorobutane	79	711	1,232
cis-3-Chloroacrylic acid	ND[b]	<10	<10
trans-3-Chloroacrylic acid	230	<10	<10

[a]Halide production at 30°C with different substrates (5 mM) was determined at pH 8.2.
[b]ND, not determined, because the enzyme is not expressed under the growth conditions used.

Mutants affected in haloalkane utilization

Strain 170 could not utilize 1,2-dibromoethane, although its dehalogenase was able to catalyze conversion of 1,2-dibromoethane to 2-bromoethanol and hydrolysis of the latter to ethylene glycol. 1,2-Dibromoethane was not used for growth since ethylene glycol did not support growth and because the intermediate 2-bromoethanol was oxidatively converted to a toxic product, presumably 2-bromoacetaldehyde. Therefore, we could use 1,2-dibromoethane as a suicide substrate to select for resistant mutants that were expected to have lost their dehalogenase activity.

Strain 170M4 was isolated by selecting for 1,2-dibromoethane resistance of strain 170 on MMY plates containing 5 mM *cis*-3-chloroacrylic acid and 20 µl of 1,2-dibromoethane in the lid of each petri dish. The mutant was not able to utilize 1,3-dichloropropene, 1-chloropropane, 1-chlorobutane, or 1-chloropentane as a sole carbon source. Growth on 3-chloroallyl alcohol and 3-chloroacrylic acid was not affected compared with wild type growth.

Crude extracts prepared from 170M4 cells did not exhibit dehalogenase activity when 1,3-dichloropropene, 1,2-dibromoethane, 1-chloropropane, or 1-chlorobutane was the substrate, but dehalogenase activity with *cis*- and *trans*-3-chloroacrylic acid was present. A

crude extract of strain 170 grown under the same conditions clearly exhibited haloalkane dehalogenase activity. Thus, strain 170M4 is defective in haloalkane dehalogenase activity and therefore is not able to utilize haloalkanes for growth.

To determine whether the structural dehalogenase gene in mutant strain 170M4 was deleted, a PCR was performed with the same primers that were used to amplify the *dhaA* gene from strain 170. Electrophoresis on an agarose gel revealed that with total DNA of strain 170M4 no DNA product was formed by PCR. The absence of the *dhaA* gene in strain 170M4 was confirmed by a Southern blot analysis performed with a probe based on the cloned *dhaA* gene of strain 170.

DISCUSSION

The fate of halogenated nematocidic soil fumigants, such as 1,3-dichloropropene, is largely dependent on the ability of microorganisms to initiate degradation through dehalogenation reactions. Although biodegradation of 1,3-dichloropropene by soil bacteria has been observed (4, 3, 21), little is known about the intermediates in the process and the dehalogenating enzymes involved in biodegradation. In this work, we describe the route of 1,3-dichloropropene metabolism in *P. cichorii* 170, an organism isolated by Verhagen and coworkers from soil that exhibited accelerated biodegradation of 1,3-dichloropropene (24).

The first step in 1,3-dichloropropene metabolism in strain 170 was catalyzed by a hydrolytic haloalkane dehalogenase with broad substrate specificity (Fig. 3). This enzyme is different from the *cis*- and *trans*-3-chloroacrylic acid dehalogenases, as shown by substrate assays performed with purified haloalkane dehalogenase. Its involvement in the metabolism of several halogenated compounds was evident from the absence of the enzyme in a mutant that was impaired in utilization of haloalkanes and haloalkenes. PCR amplification of the haloalkane dehalogenase gene from strain 170 by using primers based on the expected similarity to the sequence of the *dhaA* gene of *R. rhodochrous* NCIMB13064 (5, 11), followed by DNA sequencing, indeed revealed a sequence identical to that of the *dhaA* gene of *R. rhodochrous* NCIMB13064. Only the 13-bp sequence corresponding to the N-terminal part of the dehalogenase was not sequenced since it was encoded by the forward primer. The N-terminal amino acid sequences were identical, however.

Further conversion of the chloroallyl alcohols produced may proceed via oxidation to chloroacrylic acids. This part of the degradation route is well known to occur in other organisms. Oxidation of *cis*- and *trans*-3-chloroallyl alcohols by cell suspensions of a *Pseudomonas* strain isolated from soil also led to the production of the corresponding chloroacrylic acids (1). Van der Waarde and coworkers (20) showed that in crude extracts of 2-chloroallyl alcohol-grown *Pseudomonas* cells, 2-chloroallyl alcohol and 3-chloroallyl

alcohol are rapidly oxidized to their corresponding chloroacrylic acids without dechlorination taking place.

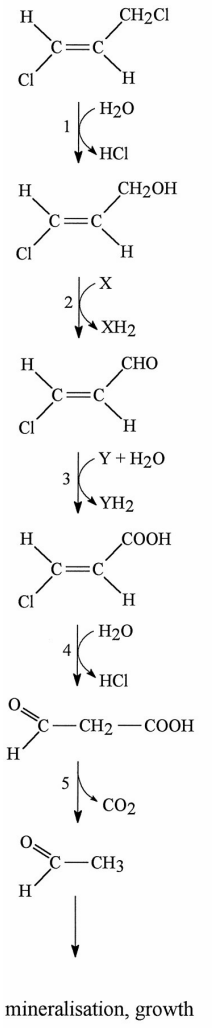

Figure 3. Proposed pathway for the degradation of *trans*-1,3-dichloropropene in *P. cichorii* 170. 1, haloalkane dehalogenase (DhaA); 2-alcohol dehydrogenase; 3, aldehyde dehydrogenase; 4, 3-chloroacrylic acid dehalogenase; 5, decarboxylase. A similar pathway is envisaged for the *cis* isomer.

The cofactor-independent dechlorination of *cis*- and *trans*-3-chloroacrylic acid in strain 170 may be catalyzed by enzymes similar to the enzymes present in the gram-positive coryneform bacterial strains CAA2 (6) and FG41 (22). These enzymes are also completely isomer selective and produce malonate semialdehyde as a product of cofactor-independent dehalogenation of 3-chloroacrylic acid. The degradative pathway of the malonate semialdehyde intermediate was studied in detail by Hartmans et al. (6). The results of these workers indicated that a cofactor-independent malonate semialdehyde decarboxylase was involved in the production of acetaldehyde and CO_2.

The massive amounts of 1,3-dichloropropene that have been applied worldwide have placed severe selective stress on bacterial populations. This probably led to fast adaptation of microorganisms to this new substrate and may have played an important role in the distribution of dehalogenase genes among different soil bacteria. Our results strongly suggest that horizontal gene transfer between gram-positive and gram-negative organisms occurs under natural conditions and may play a role in the evolution of strains adapted to degrade dichloropropenes. The molecular mechanism underlying the spread of this gene between gram-positive and gram-negative organisms is under investigation. The 1,3-dichloropropene degrading organisms that have been isolated may have evolved from organisms capable of utilizing allylalcohol or other alcohols. Rapid biodegradation of 3-chloroallyl alcohol has been observed previously, and this compound is a good growth substrate for many bacteria (1, 20). Possibly, the haloalkane dehalogenase gene was transferred to a 3-chloroallyl alcohol degrading organism, which allowed it to grow directly on 1,3-dichloropropene or made it more resistant to this toxic compound.

ACKNOWLEDGMENTS

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Chapter 3

Degradation of 1,2-dibromoethane by *Mycobacterium* sp. strain GP1

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The newly isolated bacterial strain GP1 can utilize 1,2-dibromoethane as the sole carbon and energy source. On the basis of 16S rRNA gene sequence analysis, the organism was identified as a member of the subgroup which contains the fast-growing mycobacteria. The first step in 1,2-dibromoethane metabolism is catalyzed by a hydrolytic haloalkane dehalogenase. The resulting 2-bromoethanol is rapidly converted to ethylene oxide by a haloalcohol dehalogenase, in this way preventing the accumulation of 2-bromoethanol and 2-bromoacetaldehyde as toxic intermediates. Ethylene oxide can serve as a growth substrate for strain GP1, but the pathway(s) by which it is further metabolized is still unclear. Strain GP1 can also utilize 1-chloropropane, 1-bromopropane, 2-bromoethanol, and 2-chloroethanol as growth substrates. 2-Chloroethanol and 2-bromoethanol are metabolized via ethylene oxide, which for both haloalcohols is a novel way to remove the halide without going through the corresponding acetaldehyde intermediate. The haloalkane dehalogenase gene was cloned and sequenced. The dehalogenase ($DhaA_f$) encoded by this gene is identical to the haloalkane dehalogenase (DhaA) of *Rhodococcus rhodochrous* NCIMB13064, except for three amino acid substitutions and a 14-amino-acid extension at the C terminus. Alignments of the complete dehalogenase gene region of strain GP1 with DNA sequences in different databases showed that a large part of a *dhaA* gene region, which is also present in *R. rhodochrous* NCIMB13064, was fused to a fragment of a haloalcohol dehalogenase gene that was identical to the last 42 nucleotides of the *hheB* gene found in *Corynebacterium* sp. strain N-1074.

INTRODUCTION

1,2-Dibromoethane is a synthetic organic chemical that was used primarily in an antiknock additive to gasoline. It is also one of the most effective and widely used pesticidal soil fumigants. Reduction in the use of leaded gasoline since the late 1970s and of 1,2-dibromoethane for agricultural applications in the late 1980s, owing to its cancer-causing potential and its detection in groundwater supplies, has reduced human exposure to this extremely toxic xenobiotic. However, it is still produced in large amounts for use as a lead scavenger in some countries; as a fumigant for stored grain; as a solvent for resins, gums and waxes; and as an intermediate in the synthesis of dyes and pharmaceuticals (1).

Many years after its last known application as a soil fumigant, residual 1,2-dibromoethane is still found at remarkably high concentrations in soil because it strongly interacts with the soil matrix (37). 1,2-Dibromoethane can slowly leach from such contaminated soils to groundwater over exceedingly long periods, and because of its slow chemical conversion in aqueous milieu, it is a continuous source of contamination of water supplies.

For a better understanding of the fate and persistence of 1,2-dibromoethane in the environment and for the development of bioremediation techniques for the cleanup of polluted locations, it is important to study the physiology and ecology of bacteria that degrade this toxic compound. Although biodegradation of 1,2-dibromoethane in soil under aerobic and anaerobic conditions was demonstrated by different researchers (5, 7, 8, 26, 27, 37), little is known about the biology of the bacteria that catalyze these reactions, because attempts to obtain pure cultures of bacteria that can metabolize 1,2-dibromoethane have been unsuccessful up to now.

The first report concerning the enrichment and isolation of 1,2-dibromoethane-degrading organisms was published recently (14). In this report, Freitas dos Santos et al. described the enrichment of a mixed bacterial culture capable of complete aerobic mineralization of 1,2-dibromoethane. Here we describe the isolation of a pure bacterial culture that can utilize 1,2-dibromoethane as a sole carbon and energy source. It was obtained by using the mixed culture described by Freitas dos Santos et al. (14) as an inoculum in our further isolation experiments. The results demonstrate that the newly isolated organism belongs to the genus *Mycobacterium* and metabolizes 1,2-dibromoethane via ethylene oxide by the sequential action of a hydrolytic haloalkane dehalogenase and a haloalcohol dehalogenase. The haloalkane dehalogenase gene was isolated from a cosmid library, and its nucleotide sequence and deduced amino acid sequence were compared with sequences in different DNA and protein databases.

MATERIALS AND METHODS

Chemicals and enzymes

All halogenated compounds were supplied by Acros Organics (Geel, Belgium) and were at least 97% pure according to the manufacturer. Ethylene oxide was obtained from Hoek Loos (Schiedam, The Netherlands). Restriction enzymes, T4-DNA ligase, *Taq* DNA polymerase, standard *Taq* amplification buffer, the DNA-packaging kit, and chemicals used for PCR amplification were purchased from Boehringer (Mannheim, Germany).

Bacterial strains, plasmids, and growth conditions

Strain GP1 was isolated from the mixed bacterial culture, capable of aerobic degradation of 1,2-dibromoethane, described by Freitas dos Santos et al. (14). Samples of the mixed culture (5%, vol/vol) were transferred to mineral medium (MMY) supplemented with 2 mM 1,2-dibromoethane. Prolonged batch enrichment was carried out without shaking at room temperature, after which organisms were isolated on MMY agar plates that were incubated with 7.5 μl of 1,2-dibromoethane on a filter in the lid of each petri dish. After 5 and 10 days of incubation at 30°C, 7.5 μl of 1,2-dibromoethane was again added to each filter.

MMY contained (per liter) 5.4 g of $Na_2HPO_4.12H_2O$, 1.4 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4.7H_2O$, 10 mg of yeast extract, and 5 ml of salts solution (16). Carbon sources were added up to 5 mM, calculated as if the compounds added were completely dissolved in the water phase. Cells of strain GP1 were grown in serum flasks that were filled to one-fifth of their volume and were closed gas-tight with Teflon-lined screw caps to prevent evaporation of volatile substrates. The cultures were incubated at 25ºC under rotary shaking, and growth was monitored turbidimetrically at 450 nm.

Yeast extract-peptone-dextrose (YEPD) agar (containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and MMY supplemented with 5 mM *n*-propanol were used as multipurpose growth media for strain GP1. *Escherichia coli* JM101 and plasmid pCR2.1 (Invitrogen, Leek, The Netherlands) were used for cloning of PCR products. *E. coli* HB101 was used as the recipient in transduction experiments. Plasmids pLAFR3 (36) and pBluescript SK^- (Stratagene, Leusden, The Netherlands) were used as cloning vectors. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (32). When required, Difco agar (15 g/liter) was added to the medium. LBZ medium, used for qualitative dehalogenase activity determination, was solid LB medium without NaCl. Ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) were added to the medium for detection of recombinant plasmids.

Crude extracts and enzyme assays

Cells of strain GP1 were harvested in the late exponential growth phase by centrifugation (10 min at 10,000 × *g*), washed with 1 volume of 50 mM Tris-sulfate buffer

(pH 8.2), and disrupted at 4ºC in an appropriate amount of this buffer by sonication (15 s per ml of suspension at a 70-W output in a Vibra cell sonicator). A crude extract was obtained by centrifugation (45 min at 16,000 × *g*).

Haloalkane and haloalcohol dehalogenase activities were measured by incubating an appropriate amount of cell extract with 3 ml of 5 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (19). All dehalogenase activities are expressed as units per miligram; 1 U was defined as the amount of enzyme required to produce 1 μmol of halide per min. Protein concentrations were estimated with Coomassie brillant blue by using bovine serum albumin as the standard. Most enzyme assays were carried out twice, and the differences in specific activity were less than 10%.

Epoxide hydrolase and epoxide carboxylase activities were measured by monitoring the time-dependent depletion of ethylene oxide by gas chromatography. Cell extracts were prepared in 50 mM Tris-sulfate buffer (pH 8.2) containing 10% glycerol. Assays were performed in sealed 30-ml flasks by mixing 1 ml of cell extract (3 mg of total protein) with 0.2 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2). For epoxide carboxylase assays, the reagents and reaction conditions were those described previously (2, 3).

Coenzyme A (CoA)-dependent conversion of ethylene oxide by cell extracts was also measured by monitoring the time-dependent depletion of ethylene oxide by gas chromatography. Cell extracts were prepared in 50 mM sodium phosphate buffer (pH 7.2) containing 2 mM cysteine. Assays were performed in sealed 30-ml flasks by mixing 1 ml of cell extract with 0.2 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2), with reagents and reaction conditions as described previously (10). Chemical hydrolysis of ethylene oxide was negligible under the conditions used.

Gas chromatography

Ethylene oxide, 2-bromoethanol, and 2-chloroethanol were analyzed by capillary gas chromatography. Samples (1 ml) were extracted with 1 ml of diethyl ether containing 0.05 mM 1-bromohexane or 1-chlorohexane as an internal standard. Extracts were analyzed by split injection of 4-μl samples into a type HP-5 column (model HP 19091J-413; Hewlett-Packard) with helium as the carrier gas. The column was installed in a model 6890 gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector. The oven was temperature programmed as follows: 3 min isothermal at 30°C followed by an increase at 10°C/min to 120°C. The retention times for ethylene oxide, 2-bromoethanol, and 2-chloroethanol were 2.2, 5.9, and 4.2 min, respectively.

Haloalkanes and (halo)alcohols were analyzed by capillary gas chromatography as described previously (34).

Construction and screening of a genomic library

General procedures for cloning and DNA manipulation were performed essentially as described by Sambrook et al. (32). Total genomic DNA was isolated from 1-propanol grown cells of strain GP1 by a previously described procedure (29). A partial *Sau*3A DNA genomic library of strain GP1 was constructed in the cosmid vector pLAFR3. Cosmid cloning was performed by the strategy described by Staskawicz et al. (36). The vector was isolated from *E. coli* HB101 by the alkali lysis method and was purified by cesium chloride gradient centrifugation (32). Ligation mixtures were packaged in vitro with a DNA-packaging kit. *E. coli* HB101 was transduced with these packaging mixtures (32), and colonies were selected on LBZ agar plates containing tetracycline.

Restriction analysis of plasmids isolated from transduced HB101 clones showed that 8 of 10 clones tested had plasmids with inserts. Tetracycline-resistant colonies were screened for dehalogenase activity by monitoring halide production upon incubation with halogenated compounds (19). For this, a small amount of cells were incubated in a microtiter plate with 150 μl of a mixture of 5 mM 1,2-dibromoethane and 2-bromoethanol in 50 mM Tris-sulfate buffer (pH 8.2). After overnight incubation of the plate at 30°C, 100 μl of 0.25 M $NH_4Fe(SO_4)_2$ in 6 M HNO_3 followed by a drop of saturated $Hg(SCN)_2$ in ethanol were added. A red color indicated the presence of dehalogenase activity.

Subcloning of the haloalkane dehalogenase gene

Cosmid pGP1-4B5, containing the haloalkane dehalogenase gene, was digested with *Pst*I, and its fragments were ligated into the *Pst*I site of pBluescript SK^-. The ligation mixture was used to transform $CaCl_2$-competent cells of *E. coli* JM101, and transformants were plated on LBZ plates containing ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.4 mM). Ampicillin-resistant white colonies displaying haloalkane dehalogenase activity with 1,2-dibromoethane were selected. Plasmid DNA (pBS4E11) of one of these colonies was isolated, and the complete 1.7-kb *Pst*I insert was sequenced.

Cloning of the 16S rRNA gene

To clone the 16S rRNA gene of strain GP1, biomass of a single colony was directly used for PCR amplification. The synthetic oligonucleotide primers used were described by Marchesi et al. (22): 63f, CAGGCCTAACACATGCAAGTC-3'; 1387r, 5'-GGGCGG(A/T)GTGTACAAGGC-3' (numbering based on the *E. coli* 16S rRNA gene [9]). The amplification reaction mixture contained standard *Taq* amplification buffer, 250 μM (each) deoxyribonucleotide triphosphate, 0.5 μM (each) primer, biomass of strain GP1, and 2.5 U of *Taq* DNA polymerase. The cycling parameters were 95°C for 10 min followed by 30

cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, with a final elongation step of 72°C for 7 min.

PCR products were cloned into plasmid pCR2.1 as specified by the manufacturer (Invitrogen). The ligation mixture was used to transform $CaCl_2$-competent cells of *E. coli* JM101, and ampicillin-resistant white colonies were selected from X-Gal plates. Plasmid DNA was isolated and checked by restriction analysis.

Nucleotide sequencing

Inserts of pBluescript SK^- and pCR2.1 were cycle sequenced with the Amersham Thermo Sequenase cycle-sequencing kit with 7-deaza-dGTP and Cy5 labelled fluorescent primers. Sequence reaction mixtures were run on the Pharmacia ALF-Express automatic sequencing machine. Both strands were sequenced to ensure accuracy.

Phylogenetic analysis

The 16S rRNA gene sequence of strain GP1 was compared with those in the GenBank database (6) by using FASTA3 (25) and with those in the Ribosomal Database Project by using the SIMILARITY RANK program (21), to determine its most similar sequences. Similar 16S rRNA gene sequences were downloaded and aligned by using CLUSTALW (38). Evolutionary distances were calculated by using the Jukes-Cantor algorithm (18), and the phylogenetic tree was determined by the neighbor-joining method (33) with TREECON for Windows (40). A sequence from *Rhodococcus rhodochrous* was used as the outgroup. Tree topologies were also compared between trees constructed by the methods of maximum likelihood and maximum parsimony by using PHYLIP version 3.5c (13) and the neighbor-joining method. Bootstrap analysis (12) of up to 500 replicates was performed on the phylogeny.

The secondary structure of the 16S rRNA molecule was analyzed with RNAVIZ (11) and the secondary-structure information at the small-subunit rRNA database at Antwerp (41) and was used to manually edit the alignment.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence and the sequence of the haloalkane dehalogenase gene region of strain GP1 have been deposited at the EMBL Nucleotide Sequence Database (accession no. AJ012626 and AJ012627, respectively).

RESULTS

Isolation and characterization of strain GP1

Recently, Freitas dos Santos et al. (14) described aerobic mineralization of 1,2-dibromoethane by a mixed bacterial culture. To isolate a pure culture capable of growth with 1,2-dibromoethane as the sole carbon and energy source, samples from this mixed culture were transferred to flasks containing MMY and scored for growth on 2 mM 1,2-dibromoethane. Initially, it took 2 weeks before growth on 1,2-dibromoethane was observed. This period was reduced to 1 day during repeated subculturing over a 12-month period, after which cells were streaked on MMY agar plates incubated in the presence of 1,2-dibromoethane in air. Individual colonies were restreaked on the same medium and tested for growth on 1,2-dibromoethane in liquid cultures. In this way, a pure culture of strain GP1 was obtained.

Strain GP1 was a gram-positive, nonmotile, oxidase-negative, and catalase-positive rod with a yellow pigmentation. The organism showed optimal growth on YEPD agar at 25 to 30ºC but no growth at 37 or 45ºC. Incubations at 30ºC yielded visible colonies after 36 to 48 h. No growth was observed on LB medium, and growth on nutrient broth and brain heart infusion medium was very poor.

Strain GP1 was able to grow on 1,2-dibromoethane, both in liquid cultures and on plates. The organism could be maintained on MMY-propanol plates for 10 serial transfers without loss of its 1,2-dibromoethane degrading capacity. Apart from halogenated compounds, a number of organic chemicals could also support growth: ethanol, 1-propanol, 1-butanol, 1-hexanol, glycerol, pyruvate, glucose, fructose, and ethylene oxide. The latter compound could serve as a growth substrate up to a concentration of at least 2.5 mM. The organism did not utilize methanol, 2-propanol, 2-butanol, ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, citrate, galactose, sucrose, maltose, acetone, acetaldehyde, *n*-pentane, *n*-hexane, benzene, or toluene.

16S rRNA gene sequence analysis

The newly determined sequence of the 1,314-bp DNA segment of the 16S rRNA gene of strain GP1 was compared with other 16S rRNA gene sequences available in the GenBank database and in the Ribosomal Database Project. From this initial screening, it was evident that strain GP1 is a *Mycobacterium* species. Optimal linear alignment results showed that strain GP1 was most similar (2 bp were different in the 1,314-bp overlap region) to *Mycobacterium* sp. strain PAH135 (15, 43). The phylogenetic tree for the 16S rRNA gene sequences showed that strain GP1 is a member of the subgroup which contains the fast-growing mycobacteria (Fig. 1). The similarity values between strain GP1 and the fast-growing mycobacteria range from 0.9452 to 0.9985. Comparison of the trees constructed by the

methods of maximum likelihood and maximum parsimony, and by the neighbor-joining method showed the same topology.

Secondary structure analysis of 16S rRNA molecules led to the identification of structural signatures, based on the length and sequence variations of two specific 16S rRNA regions, that have been proposed as identifiers for subgroups of mycobacteria (28, 31, 35). The helix flanked by positions 451 to 482 (*E. coli* numbering) differentiates the fast-growing mycobacteria from the slow-growing mycobacteria. *Mycobacterium* sp. strain GP1 has a signature identical to that of *M. komossense* (Fig. 2a), which is a representative fast grower. A second signature flanked by positions 184 to 193 (*E. coli* numbering) differentiates the thermosensitive strains from the thermotolerant strains among the fast-growing mycobacteria. *Mycobacterium* sp. strain GP1 shared the signature proposed for thermosensitive strains, which is in agreement with the physiological data obtained for strain GP1. Its signature is similar in secondary structure, but not in primary structure, to *M. komossense* (Fig. 2b).

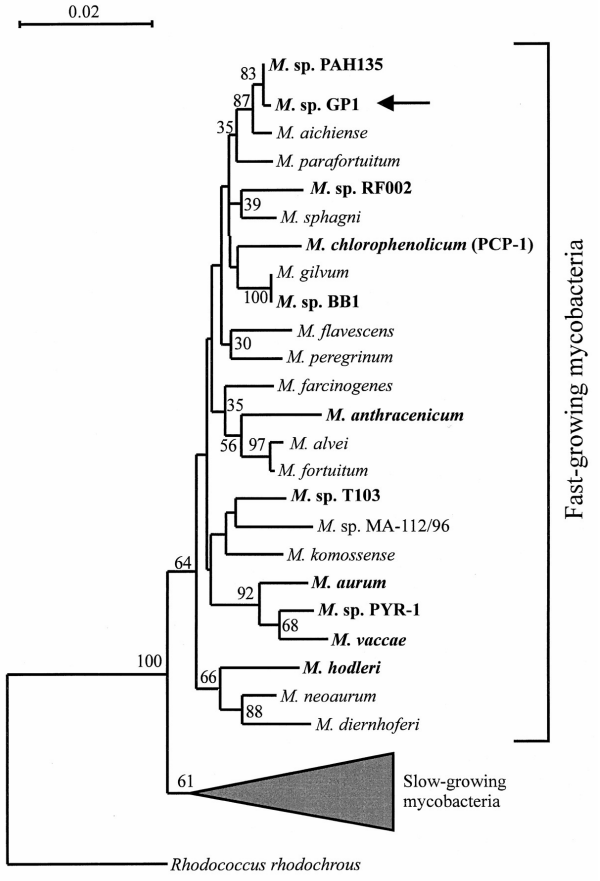

Figure 1. Phylogenetic tree based on 16S rRNA gene sequence analysis, illustrating the relationships of strain GP1 to the most closely related bacteria. Base positions 54 to 1368 (numbering based on the *E. coli* 16S rRNA gene) were included in the analysis. The scale bar represents 0.02 fixed mutation per site. Bootstrap values were derived from 500 analyses. All organisms with known biodegradative capabilities are shown in boldface.

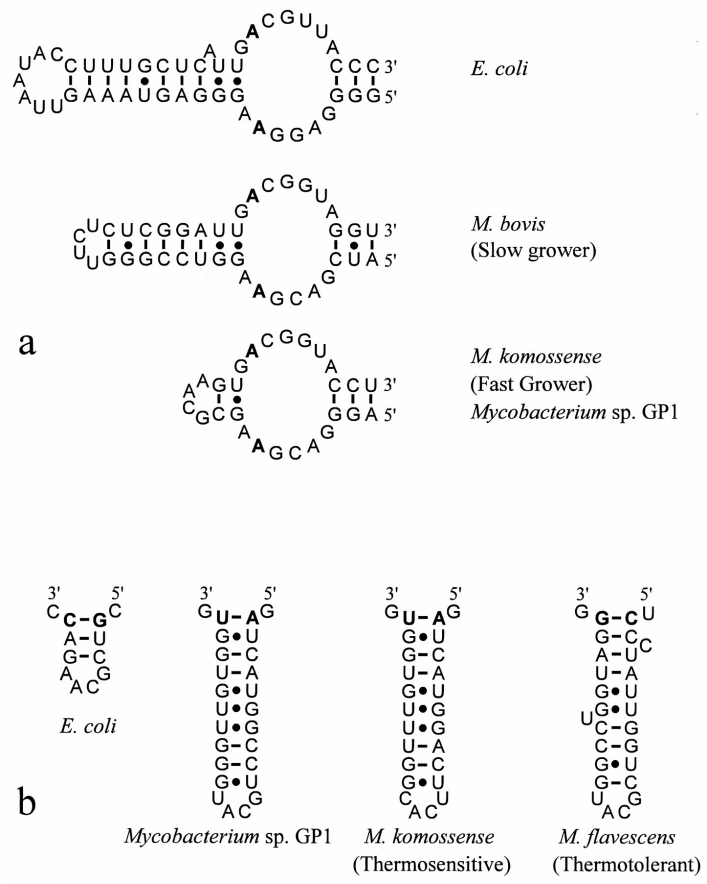

Figure 2. Comparison of secondary structures of the 16S rRNA molecule. (a) Signatures in the V3 variable region which differentiate between the fast- and slow-growing mycobacteria. Representatives of both classes are shown. Bases 451 and 482 (from the 5' end) are shown in boldface. (b) Signatures in the V2 variable region which differentiate between the thermotolerant and thermosensitive mycobacteria. Representatives of both classes are shown. Bases 184 and 193 (from the 5' end) are shown in boldface.

Utilization of halogenated compounds

Growth of strain GP1 with 2 mM 1,2-dibromoethane as the substrate was monitored in batch culture (Fig. 3). Growth resulted in disappearance of the substrate and simultaneous formation of biomass and inorganic bromide, with no indication of the accumulation of brominated intermediates. Ethylene oxide, however, transiently accumulated in the medium, indicating that this is an intermediate formed during the degradation of 1,2-dibromoethane. Concentrations of 1,2-dibromoethane above 2.5 mM were toxic for strain GP1 and completely inhibited growth on pyruvate or fructose.

We determined whether strain GP1 could utilize halogenated compounds that are structurally related to 1,2-dibromoethane and its possible degradation products. The results showed that besides 1,2-dibromoethane, only a few halogenated compounds support growth (Table 1). 1-Bromopropane, 1-chloropropane, and 2-chloroethanol could serve as growth substrates up to a concentration of at least 5 mM, whereas concentrations of 2-bromoethanol

above 1.5 mM were very toxic for strain GP1 and completely inhibited growth. No growth was observed with 1 mM dibromomethane, dichloromethane, diiodomethane, bromochloromethane, bromoform, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,3-dibromopropane, 1,3-dichloropropane, 1,2,3-trichloropropane, 1,2,3-tribromopropane, 1,2-dibromo-3-chloropropane, 2-bromo-1-chloropropane, 1-bromo-3-chloropropane, 1-chlorobutane, 1-chloropentane, 1-chlorohexane, 1-bromohexane, 1-bromodecane, 1,3-dichloropropene, 1,2-dichloroethylene, bromoacetate, chloroacetate, dibromoacetate, dichloroacetate, 2-chloropropionic acid, 3-chloropropionic acid, 3-chloro-1,2-propanediol, 1,3-dichloro-2-propanol, 2,3-dichloro-1-propanol, and 1-chloro-2-propanol.

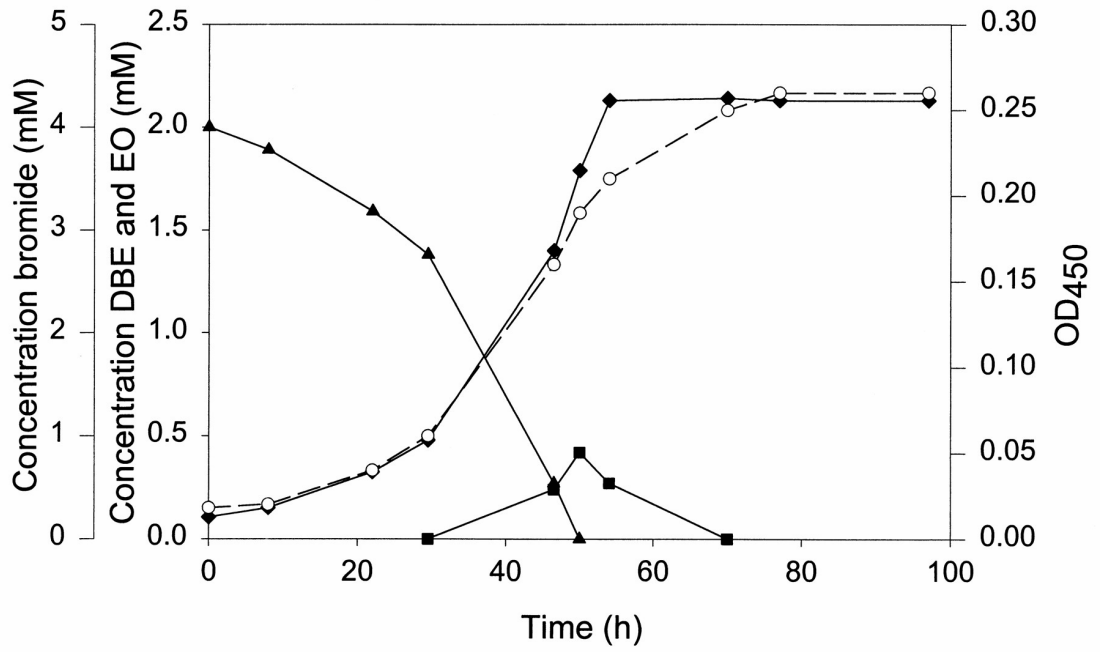

Figure 3. Growth of strain GP1 on 2 mM 1,2-dibromoethane in batch culture. Symbols: ▲, 1,2-dibromoethane (DBE) concentration; ■, ethylene oxide (EO) concentration; ◆, bromide concentration; O, optical density at 450 nm (OD_{450}).

Metabolism of 1,2-dibromoethane

Activities of enzymes that may be involved in 1,2-dibromoethane metabolism were tested with crude extracts prepared from cells grown on either 1,2-dibromoethane or 1-propanol (Table 2). Extract of 1,2-dibromoethane-grown cells converted 1,2-dibromoethane, bromochloroethane, 1-chloropropane, and 1-bromobutane to the corresponding monoalcohols and halide ions, which indicates that dehalogenation of haloalkanes is a hydrolytic reaction in this organism. There was no significant difference in haloalkane dehalogenase activities in extracts prepared from 1-propanol-grown cells and those in extracts prepared from 1,2-

dibromoethane-grown cells, indicating that expression of the haloalkane dehalogenase is constitutive and independent of the growth substrate used. The highest level of haloalkane dehalogenase activity was observed with 1,2-dibromoethane, whereas low dehalogenase activity (less than 30 mU/mg) was measured with the analog 1,2-dichloroethane.

Table 1. Utilization of halogenated compounds by strain GP1

Carbon source	Conc (mM)	Generation time[b] (h)	Halide production[a] (mM) in:	
			Inoculated medium	Sterile control
1-Bromopropane	2	42	2.1	0.1
1-Chloropropane	2	50	2.0	<0.1
2-Bromoethanol	1	18	1.0	0.15
2-Chloroethanol	2	20	2.0	<0.1

[a]Halide levels were determined after 2 weeks of cultivation in liquid MMY at 25ºC.
[b]Generation times were determined after inoculation (1%, vol/vol) of fresh medium with cells from a culture grown on the same carbon source.

Conversion of the hydrolytic product of 1,2-dibromoethane, 2-bromoethanol, was initially expected to proceed via two oxidation steps to bromoacetate, which may then be dehalogenated by a haloacid dehalogenase. This degradation route is known to occur in 1,2-dichloroethane-degrading *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* strains (17). However, extracts of 1,2-dibromoethane- or 1-propanol-grown cells showed no dehalogenase activity toward bromoacetate or other halogenated carboxylic acids tested (Table 2).

Surprisingly, rapid halide production was observed upon incubation of extracts with 2-bromoethanol and several other haloalcohols tested, indicating that direct dehalogenation of haloalcohols does occur (Table 2). The product formed from 2-bromoethanol (Fig. 4) and 2-chloroethanol was identified as ethylene oxide, whereas 1,3-dichloro-2-propanol and 1-chloro-2-propanol were converted to epichlorohydrin and propylene oxide, respectively. This haloalcohol dehalogenase activity was slightly elevated in extracts prepared from 1-propanol-grown cells compared to that in extracts prepared from 1,2-dibromoethane-grown cells. The highest level of haloalcohol dehalogenase activity was observed with 2-bromoethanol, indicating that this toxic intermediate was rapidly converted to the less toxic ethylene oxide.

The formation of different products during the conversion of haloalkanes and haloalcohols suggests that at least two different dehalogenating enzymes are present in strain

GP1: a hydrolytic haloalkane dehalogenase, which converts haloalkanes to the corresponding (halo)alcohols and halide ions, and a haloalcohol dehalogenase (also called halohydrin hydrogen-halide lyase [23, 24]), which converts haloalcohols to the corresponding epoxides and halide ions. Conversion of haloalkanes and haloalcohols to different products by a single enzyme is unlikely. Enzymes which are involved in hydrolytic conversion of haloalkanes (17, 20, 29) and haloalcohol dehalogenases that show lyase activity toward haloalcohols (23, 39) have been described previously, but strain GP1 is the first organism found to produce both dehalogenating enzymes. Together, these enzymes convert 1,2-dibromoethane to ethylene oxide.

Table 2. Dehalogenase activities in crude extracts from strain GP1

	Dehalogenase sp act[a] (mU/mg of protein)		
Substrate	1,2-DBE	1-Propanol	Product
1,2-Dibromoethane	220	230	2-Bromoethanol
1,2-Dichloroethane	<30	<30	
Bromochloroethane	180	ND[b]	2-Chloroethanol
1-Chloropropane	50	ND	1-Propanol
1-Bromobutane	30	ND	1-Butanol
1,3-Dibromopropane	80	80	ND
2-Bromoethanol	5200	8110	Ethylene oxide
2-Chloroethanol	70	110	Ethylene oxide
3-Chloro-1,2-propanediol	ND	300	ND
2,3-Dichloro-1-propanol	ND	<30	
1,3-Dichloro-2-propanol	ND	5220	Epichlorohydrin
1-Chloro-2-propanol	ND	3260	Propylene oxide
Bromoacetate	<30	<30	
Chloroacetate	<30	<30	
Dibromoacetate	<30	<30	
Dichloroacetate	<30	<30	

[a]Specific activities with various substrates (5 mM) were determined with extracts prepared from cells grown with either 1,2-dibromoethane or 1-propanol.
[b]ND, not determined.

Further metabolism of ethylene oxide is likely to proceed via malonate semialdehyde (2, 3) or via acetyl-CoA (10). However, no epoxide carboxylase activity or CoA- and NAD^+-dependent conversion of ethylene oxide could be demonstrated. The possibility that ethylene glycol was an intermediate in ethylene oxide metabolism in strain GP1 is unlikely on the basis

of two observations: no epoxide hydrolase activity toward ethylene oxide was detected in cell extracts, and strain GP1 does not utilize ethylene glycol as a growth substrate.

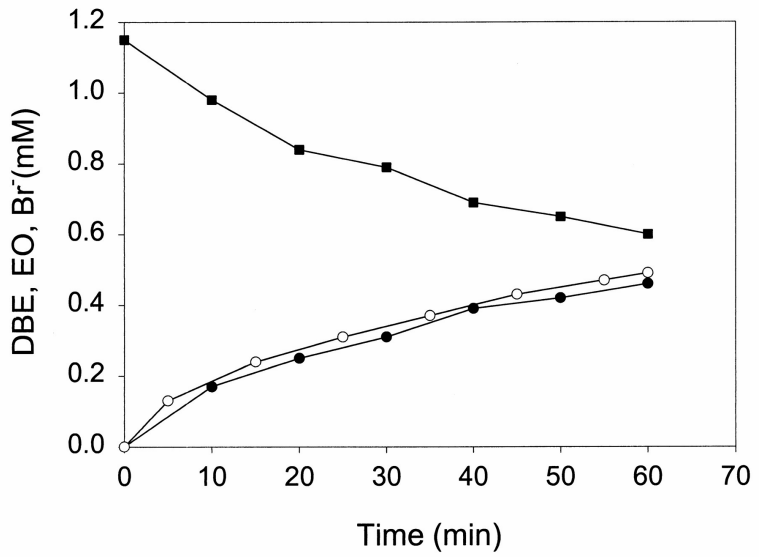

Figure 4. Dehalogenation of 2-bromoethanol (■) in crude extract of 1,2-dibromoethane-grown cells, with the production of ethylene oxide (EO) (●) and inorganic bromide (Br^-) (O).

Isolation of dehalogenase genes involved in 1,2-dibromoethane metabolism

As discussed above, the first step in 1,2-dibromoethane metabolism in strain GP1 appears to be catalyzed by a hydrolytic haloalkane dehalogenase. The activities that we measured in crude extract suggested that this enzyme resembled the haloalkane dehalogenase DhaA that was found in the gram-positive, haloalkane-degrading bacterium *R. rhodochrous* NCIMB13064 (20). However, attempts to amplify the haloalkane dehalogenase gene from strain GP1 with primers designed on the 5' and 3' ends of the reported sequence of the *dhaA* gene (20) did not result in an amplification product. These primers were successfully used to amplify the haloalkane dehalogenase gene from *Pseudomonas pavonaceae* 170 (formerly known as *P. cichorii* 170), which is 100% identical to the *dhaA* gene of strain NCIMB13064 (29). This result indicated that strain GP1 contains a haloalkane dehalogenase that is different from the previously identified DhaA in *R. rhodochrous* NCIMB13064 and *P. pavonaceae* 170.

To identify the haloalkane dehalogenase and to confirm that two different dehalogenating enzymes are involved in 1,2-dibromoethane metabolism, a gene bank of strain GP1 was constructed in the cosmid vector pLAFR3. Cosmid clones were screened for dehalogenase activity with a mixture of 1,2-dibromoethane and 2-bromoethanol, which are the best substrates for the haloalkane and haloalcohol dehalogenase, respectively. Of 1,000 clones tested, 20 dehalogenase-positive clones were found. To determine whether these expressed

haloalkane or haloalcohol dehalogenase activity, clones were incubated separately with 1,2-dibromoethane, 2-bromoethanol or 1,3-dichloro-2-propanol. All 20 clones showed halide production upon incubation with 1,2-dibromoethane, whereas none showed dehalogenase activity toward 1,3-dichloro-2-propanol. However, slow halide release was observed upon incubation of the clones with 2-bromoethanol. From these results, we concluded that all 20 dehalogenase-positive clones expressed the haloalkane dehalogenase and that this enzyme also exhibited low dehalogenase activity toward 2-bromoethanol. Hydrolytic conversion of both 1,2-dibromoethane and 2-bromoethanol was also observed for the haloalkane dehalogenase DhaA that we purified from *P. pavonaceae* 170 (unpublished data). The low dehalogenase activity toward 2-bromoethanol and the fact that the haloalkane dehalogenase clone does not convert 1,3-dichloro-2-propanol is in agreement with the presence in strain GP1 of a second dehalogenase, which rapidly converts 2-bromoethanol and other haloalcohols to the corresponding epoxides. To exclude the possibility that haloalcohol dehalogenase activity was not found in the initial screening because of inhibition of the enzyme by 1,2-dibromoethane, the complete gene library was also screened for haloalcohol dehalogenase activity with 1,3-dichloro-2-propanol. *E. coli* HB101 clones that expressed the haloalcohol dehalogenase were also not found in this screening.

Sequence of the haloalkane dehalogenase region

Plasmid pGP1-4B5 encoding haloalkane dehalogenase activity was isolated from an HB101 clone. *Pst*I fragments of this plasmid were ligated into pBluescript SK^-, and the ligation mixture was used to transform *E. coli* JM101. By using a colony assay, transformants could be tested quickly for the presence of the dehalogenase gene. Three dehalogenase-positive transformants were selected, and the corresponding plasmids all contained a single 1.75-kb *Pst*I insert. One such plasmid (pBS4E11) was used for sequencing of the dehalogenase gene.

The nucleotide sequence of the entire 1.75-kb *Pst*I fragment was determined independently in both directions (Fig. 5). An open reading frame coding for a protein of 307 amino acids was found. A search with the BLASTP program (4) in different protein databases showed that the first 293 amino acids of the deduced amino acid sequence were identical to the haloalkane dehalogenase DhaA from *R. rhodochrous* NCIMB13064 (20), except for three amino acid substitutions (Fig. 5), whereas the last 14 amino acids were identical to the C-terminal sequence of the haloalcohol dehalogenase HheB (halohydrin hydrogen-halide [H]-lyase B) from *Corynebacterium* sp. strain N-1074 (44).

To examine the apparent fusion between DhaA- and HheB-encoding sequences in more detail, the complete sequence of the haloalkane dehalogenase gene region was compared with DNA sequences in different databases by using the BLASTN search program (4). The results of this search showed that a large segment of the *dhaA* gene region, which is also present in *R. rhodochrous* NCIMB13064 (accession no. AF060871, AF017179, and L49435), was fused to

a fragment of the haloalcohol dehalogenase gene *hheB* (accession no. D90350), which was first found in *Corynebacterium* sp. strain N-1074. The fusion of the *dhaA* gene to the last 42 nucleotides of the *hheB* gene gave rise to a new open reading frame coding for a novel dehalogenase of 307 amino acids. This haloalkane dehalogenase was named $DhaA_f$. The sequence upstream of the haloalkane dehalogenase gene (*dhaA$_f$*) from strain GP1 was identical to the sequence upstream of the *dhaA* gene from *R. rhodochrous* NCIMB13064 except for a small deletion of 12 nucleotides (Fig. 5). The sequence downstream of *dhaA$_f$* was 100% identical to the reported sequence downstream of *hheB* (44). To eliminate the possibility that the fusion of the *dhaA* gene region to the *hheB* gene occurred upon (sub)cloning, the fusion was confirmed by PCR analysis with total DNA of strain GP1 as the template (results not shown).

DISCUSSION

The fate and persistence of many pesticidal soil fumigants in the environment is largely dependent on the ability of microorganisms to metabolize these compounds. Although slow biodegradation of the soil fumigant and priority pollutant 1,2-dibromoethane by soil bacteria has been observed (5, 7, 8, 26, 27, 37), little is known about the intermediates in the process and the enzymes involved in biodegradation. In this paper, we describe the properties of the newly isolated strain GP1, which is capable of aerobic degradation and utilization of 1,2-dibromoethane. To our knowledge, this is the first report that describes the utilization of 1,2-dibromoethane as a growth substrate by a pure bacterial culture.

We were able to isolate strain GP1 from a mixed bacterial culture capable of aerobic mineralization of 1,2-dibromoethane (14) by prolonged batch enrichment. Attempts to isolate single 1,2-dibromoethane-degrading organisms from the mixed culture, without any adaptation procedure, were unsuccessful. The long period of selection (15 to 20 subcultivations) necessary to obtain a pure culture of strain GP1 suggests that this strain obtained or improved its 1,2-dibromoethane-degrading capacity during batch enrichment.

Strain GP1 was identified as a member of the subgroup which contains the thermosensitive, fast-growing mycobacteria. The physiological data obtained for strain GP1 and its fatty acid profile (LMG culture collection, Gent, Belgium [data not shown]) support this identification. Strain GP1 is the first member of the genus *Mycobacterium* that is capable of degrading short-chain haloalkanes.

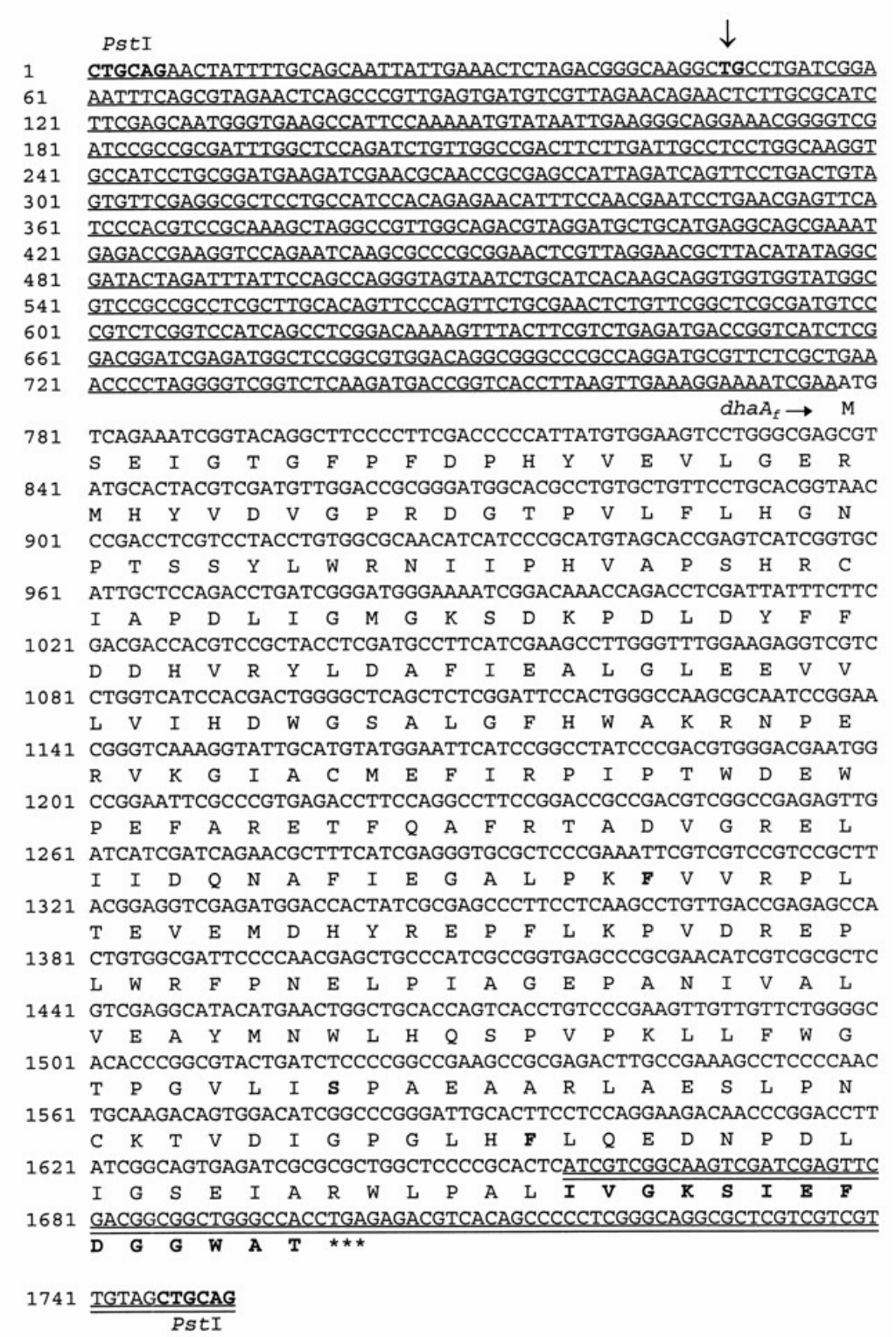

Figure 5. Complete nucleotide sequence of the 1.75-kb *Pst*I fragment containing the haloalkane dehalogenase gene. The deduced amino acid sequence of the dehalogenase open reading frame is indicated below the corresponding DNA sequence. The three amino acid substitutions and the 14-amino-acid extension at the C terminus, which are the differences compared to the DhaA amino acid sequence, are shown in boldface. The sequence upstream of $dhaA_f$ identical to the sequence upstream of the *dhaA* gene in *R. rhodochrous* NCIMB13064 is underlined. The position of the deletion of 12 nucleotides, compared to the *dhaA* gene region in strain NCIMB13064, between nucleotides 49 (**T**) and 50 (**G**) is indicated by an arrow. The sequence identical to the *hheB* gene region in *Corynebacterium* sp. strain N-1074 is indicated by a double underline.

1,2-Dibromoethane is an excellent substrate for the haloalkane dehalogenase (DhlA) present in the 1,2-dichloroethane-degrading *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* strains (17). The resulting 2-bromoethanol is expected to be oxidized in two steps to bromoacetate, which can be rapidly hydrolyzed by a haloacid dehalogenase (Fig. 6). However, these organisms cannot utilize 1,2-dibromoethane, because both 1,2-dibromoethane and 2-bromoethanol are toxic for these strains in the micromolar range. The absence of a functional aldehyde dehydrogenase, which results in the accumulation of the highly reactive bromoacetaldehyde, seems to be the cause of the lack of utilization of 1,2-dibromoethane for growth by 1,2-dichloroethane-degrading bacteria (42).

Mycobacterium sp. strain GP1 metabolizes 1,2-dibromoethane via ethylene oxide by the sequential action of a hydrolytic haloalkane dehalogenase and a haloalcohol dehalogenase (Fig. 6). The latter enzyme was highly active toward 2-bromoethanol and rapidly converted this intermediate to ethylene oxide. In this way, the organism prevents the accumulation of the toxic intermediates 2-bromoethanol and 2-bromoacetaldehyde, which were shown to be lethal for 1,2-dichloroethane-degrading strains (17, 42). Complete metabolism of 1,2-dibromoethane thus necessitates the sequential use of two dehalogenating enzymes to prevent the formation of toxic brominated intermediates.

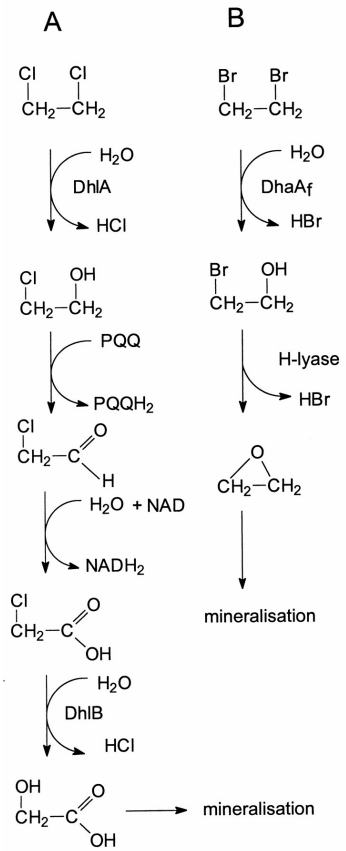

Figure 6. (A) Proposed route of the metabolism of 1,2-dichloroethane in *X. autotrophicus* and *A. aquaticus* strains. (B) Proposed route of the metabolism of 1,2-dibromoethane in *Mycobacterium* sp. strain GP1.

The pathway(s) by which the epoxide intermediate, ethylene oxide, is further metabolized in *Mycobacterium* sp. strain GP1 is still unclear. Well-known strategies for epoxide conversion, such as epoxide carboxylation (2, 3), epoxide hydration (30), or transformation to acetyl-CoA (10), could not be demonstrated for the conversion of ethylene oxide in crude extracts of strain GP1. Hydrolysis of ethylene oxide to ethylene glycol is also unlikely, since strain GP1 does not utilize ethylene glycol as a growth substrate.

Isolation of the haloalkane dehalogenase gene of strain GP1 from a cosmid library was possible by screening recombinant *E. coli* HB101 clones for dehalogenase activity toward 1,2-dibromoethane. The dehalogenase gene appeared to encode a 307-amino-acid polypeptide, which is the result of a fusion between two known genes which encode dehalogenating enzymes. The first 293 amino acids were identical to the complete haloalkane dehalogenase DhaA (20), except for three amino acid substitutions, whereas the last 14 amino acids of the deduced amino acid sequence were identical to the C-terminal sequence of the haloalcohol dehalogenase HheB from *Corynebacterium* sp. strain N-1074 (44). Whether the fusion of the *dhaA* gene region to the *hheB* gene region, giving rise to a new open reading frame of 307 amino acids, occurred during prolonged enrichment because of the selective advantage provided by the new haloalkane dehalogenase or whether this is coincidental is unclear. The enzymatic activities of $DhaA_f$ and DhaA seem very similar, indicating that the three amino acid substitutions and the 14-amino-acid extension have no important influence on dehalogenating capacity. In either case, it is surprising that the haloalkane dehalogenase gene was fused exactly to the *hheB* gene, which normally encodes an enzyme that could be involved in the metabolism of the compound used for selection. This could reflect a preference for recombination events in the *hheB* gene region, which is under selective pressure.

The cofactor-independent debromination of 2-bromoethanol in strain GP1 may be catalyzed by an enzyme similar to the haloalcohol dehalogenases (H-lyases) present in *Corynebacterium* sp. strain N-1074 (HheB) (23, 24) and in *Arthrobacter* sp. strain AD2 (39). These enzymes also catalyze the conversion of haloalcohols (halohydrins) to the corresponding epoxides and halide ions. However, crude extract of strain GP1 has a very high specific dehalogenase activity toward 2-bromoethanol (5,000 to 8,000 mU/mg of protein), which is comparable to the specific dehalogenase activities measured for the purified haloalcohol dehalogenases from strains N-1074 and AD2 (11,000 and 2,000 mU/mg of protein, respectively). Surprisingly, no abundant protein band was observed when a crude extract of 1,2-dibromoethane-grown cells was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These results suggest that haloalcohol dehalogenase of strain GP1 is capable of degrading 2-bromoethanol much faster than are the dehalogenases found in strains N-1074 and AD2. This is in agreement with the observation that the rapid dehalogenation of 2-bromoethanol is an important step in the metabolism of 1,2-dibromoethane.

ACKNOWLEDGMENTS

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Chapter 4

Roles of horizontal gene transfer and gene integration in evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways

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The haloalkane-degrading bacteria *Rhodococcus rhodochrous* NCIMB13064, *Pseudomonas pavonaceae* 170, and *Mycobacterium* sp. strain GP1 share a highly conserved haloalkane dehalogenase gene (*dhaA*). Here, we describe the extent of the conserved *dhaA* segments in these three phylogenetically distinct bacteria and an analysis of their flanking sequences. The *dhaA* gene of the 1-chlorobutane-degrading strain NCIMB13064 was found to reside within a 1-chlorobutane catabolic gene cluster, which also encodes a putative invertase (*invA*), a regulatory protein (*dhaR*), an alcohol dehydrogenase (*adhA*), and an aldehyde dehydrogenase (*aldA*). The latter two enzymes may catalyze the oxidative conversion of *n*-butanol, the hydrolytic product of 1-chlorobutane, to *n*-butyric acid, a growth substrate for many bacteria. The activity of the *dhaR* gene product was analyzed in *Pseudomonas* sp. strain GJ1, in which it appeared to function as a repressor of *dhaA* expression. The 1,2-dibromoethane-degrading strain GP1 contained a conserved DNA segment of 2.7 kb, which included *dhaR*, *dhaA*, and part of *invA*. A 12-nucleotide deletion in *dhaR* led to constitutive expression of *dhaA* in strain GP1, in contrast to the inducible expression of *dhaA* in strain NCIMB13064. The 1,3-dichloropropene-degrading strain 170 possessed a conserved DNA segment of 1.3 kb harboring little more than the coding region of the *dhaA* gene. In strains 170 and GP1, a putative integrase gene was found next to the conserved *dhaA* segment, which suggests that integration events were responsible for the acquisition of these DNA segments. The data indicate that horizontal gene transfer and integrase-dependent gene acquisition were the key mechanisms for the evolution of catabolic pathways for the man-made chemicals 1,3-dichloropropene and 1,2-dibromoethane.

INTRODUCTION

Synthetic haloalkanes form an important class of environmental pollutants because of their widespread use in industry and agriculture, persistence in the environment, and potential carcinogenicity. The poor biodegradability of these chemicals is mainly due to the inability of microorganisms to effectively metabolize these unnatural compounds. Nevertheless, microbial communities exposed to synthetic haloalkanes often respond by expressing specific pathways that degrade these molecules in order to exploit them as growth substrates. Since synthetic haloalkanes are xenobiotic compounds of recent origin, the way in which genes have been assembled to form functional catabolic pathways is an interesting subject for studying microbial evolution and gene transfer.

Rhodococcus rhodochrous NCIMB13064, isolated in the United Kingdom from a soil sample obtained from an industrial site which had previously been exposed to chlorinated alkanes, is capable of utilizing 1-chlorobutane and several other haloalkanes as the sole carbon and energy source (9). The cleavage of the carbon-halogen bond in 1-chlorobutane, which is the key step in its catabolism, is catalyzed by an inducible hydrolytic haloalkane dehalogenase (DhaA) and results in the formation of *n*-butanol. This intermediate is subsequently oxidized in two steps to *n*-butyric acid (Fig. 1), which can serve as a growth substrate for many bacteria. The haloalkane dehalogenase gene (*dhaA*) was shown to be located on the autotransmissible plasmid pRTL1 (22) and was cloned and sequenced (23).

The gram-negative 1,3-dichloropropene-utilizing bacterium *Pseudomonas pavonaceae* 170, isolated in The Netherlands from soil that was repeatedly treated with the nematocidic soil fumigant 1,3-dichloropropene, was shown by PCR amplification to possess a haloalkane dehalogenase gene identical to the *dhaA* gene of the gram-positive strain NCIMB13064 (35). In contrast to the inducible production of DhaA in strain NCIMB13064, DhaA is constitutively produced in strain 170 and catalyzes the first step in the degradation of 1,3-dichloropropene (Fig. 1).

Recently, we demonstrated that the 1,2-dibromoethane-degrading organism *Mycobacterium* sp. strain GP1, which was isolated by prolonged batch enrichment from a mixed bacterial culture, also contains a haloalkane dehalogenase gene ($dhaA_f$) that is very similar to the *dhaA* gene found in strain NCIMB13064 (36). The haloalkane dehalogenase encoded by $dhaA_f$ is identical to DhaA, except for three amino acid substitutions and a 14-amino-acid extension at the C-terminus. Nucleotide sequence analysis indicated that the $dhaA_f$ gene was formed by a fusion of a *dhaA* gene with the last 42 nucleotides of a *hheB* gene, which encodes a haloalcohol dehalogenase (50). The haloalkane dehalogenase ($DhaA_f$) is constitutively produced in strain GP1 and catalyzes the conversion of 1,2-dibromoethane to 2-bromoethanol, which is further metabolized via ethylene oxide (Fig. 1).

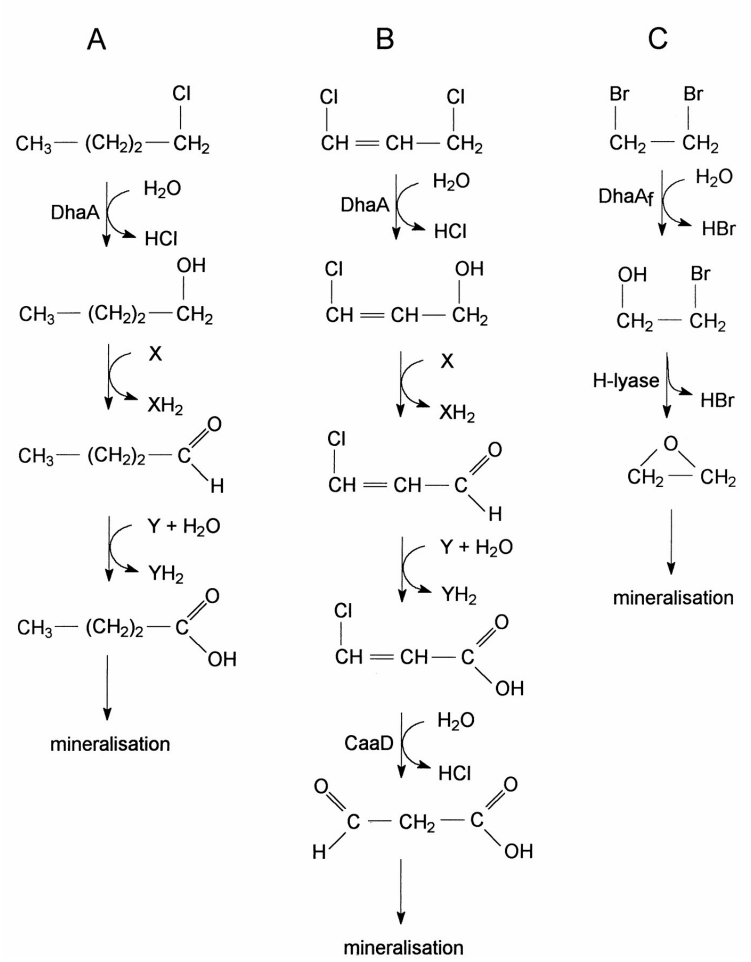

Figure 1. Catabolic pathways for haloaliphatics. (A) 1-chlorobutane in *R. rhodochrous* NCIMB13064. (B) 1,3-dichloropropene in *P. pavonaceae* 170. (C) 1,2-dibromoethane in *Mycobacterium* sp. strain GP1. Abbreviations: DhaA and $DhaA_f$, haloalkane dehalogenases; H-lyase, halohydrin halogen-halide lyase; CaaD, 3-chloroacrylic acid dehalogenase; X, alcohol dehydrogenase cofactor; Y, aldehyde dehydrogenase cofactor.

The presence of a highly conserved *dhaA* gene in the three phylogenetically different organisms *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 suggests that *dhaA* has been distributed among these organisms by horizontal transfer. To determine the size of the transferred DNA fragments and to identify the mechanisms that were involved in the distribution process, we have analyzed the DNA regions flanking the *dhaA* gene in these three haloalkane-utilizing strains. The results suggest that the haloalkane dehalogenase gene regions of strains 170 and GP1 originate from a 1-chlorobutane catabolic gene cluster similar to the one that is present on plasmid pRTL1 in strain NCIMB13064. Horizontal gene transfer and integrase-dependent acquisition of existing DNA fragments harboring the *dhaA* gene were probably the key steps during the evolution of 1,3-dichloropropene- and 1,2-dibromoethane-degradative pathways. Furthermore, the constitutive expression of *dhaA* in strains 170 and GP1, in contrast to the inducible expression of *dhaA* in strain NCIMB13064, is explained by the absence or inactivation of the regulatory gene *dhaR*.

MATERIALS AND METHODS

Materials

Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase, the DNA-packaging kit, and materials used for Southern blot hybridization were purchased from Boehringer Mannheim (Mannheim, Germany). 1,2-Dibromoethane was supplied by Acros Organics (Geel, Belgium). The oligonucleotides used as primers were supplied by Eurosequence BV (Groningen, The Netherlands).

Bacterial strains, plasmids, and growth conditions

The characteristics of the 1,3-dichloropropene-degrading bacterium *P. pavonaceae* 170, formerly known as *Pseudomonas cichorii* 170 (44), and of the 1,2-dibromoethane-degrading organism *Mycobacterium* sp. strain GP1 are given elsewhere (35, 36). *R. rhodochrous* NCIMB13064 contains the large catabolic plasmid pRTL1 (22) and is able to use 1-chlorobutane as the sole carbon and energy source (9). *Escherichia coli* strains HB101 (7) and JM101 (49) and plasmid pBluescript SK(-) (Stratagene, Leusden, The Netherlands) were used for routine cloning experiments. *E. coli* HB101(pRK600) (13) was the helper strain used for mobilizing pLAFR3/5- and pDSK519-derived plasmids in triparental matings with the recipient *Pseudomonas* sp. strain GJ1 (17). Plasmid pDSK519 and cosmids pLAFR3 and pLAFR5 are mobilizable broad-host-range vectors (19, 39). Recombinant cosmid pLTL1k contains the 1-chlorobutane catabolic gene cluster of strain NCIMB13064 (23) and was used as template for DNA sequencing and as the source for some cloning and expression experiments described here. Recombinant cosmids pGP1-4B5, which contains the $dhaA_f$ gene region of strain GP1 (36), and pPC33, which contains the *dhaA* gene region of strain 170 (this study), were used as templates for DNA sequencing.

R. rhodochrous NCIMB13064 and *Mycobacterium* sp. strain GP1 were grown at 30ºC on nutrient broth or on mineral medium (17) supplemented with 5 mM n-propanol, respectively. *Pseudomonas* and *E. coli* strains were grown at 30ºC on Luria-Bertani medium (38). When required, Difco agar (15 g/liter) was added to the medium. LBZ medium, used for qualitative dehalogenase activity determination, was solid Luria-Bertani medium without NaCl. Antibiotics were added in the following amounts: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 50 µg/ml; and tetracycline, 12.5 µg/ml. When necessary, media were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 40 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 0.4 mM).

DNA techniques

General procedures for cloning, transformation, and DNA manipulation were performed essentially as described by Sambrook et al. (38). Triparental matings were carried

out as described by Janssen et al. (18). Isolation of total genomic DNA from strains NCIMB13064, 170, and GP1 was performed according to the phenol extraction procedure described previously (35). The IS*2112*- and IS*1071*-specific probes used in hybridization experiments were obtained by PCR amplification using primers and conditions as previously decribed (21, 48). DNA fragments were purified by using the Qiaquick PCR purification kit (Qiagen). Southern blot hybridization experiments were performed as described previously (21).

Crude extracts and dehalogenase assays

Pseudomonas and *E. coli* cells were harvested in the late-exponential growth phase by centrifugation (10 min at 10,000 × *g*), were washed with 1 volume of 50 mM Tris-sulfate buffer (pH 8.2), and were disrupted at 4ºC in an appropriate amount of this buffer by sonication (10 s per ml of suspension at a 70 W output in a Vibra cell sonicator). A crude extract was obtained by centrifugation (45 min at 16,000 × *g*).

Haloalkane dehalogenase activities were measured by incubating an appropriate amount of cell extract with 3 ml of 5 mM 1,2-dibromoethane in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (20). All dehalogenase activities are expressed as units per miligram; 1 U was defined as the amount of enzyme that catalyzes the production of 1 μmol of halide per min. Protein concentrations were estimated with Coomassie brillant blue by using bovine serum albumin as the standard. Enzyme assays were carried out twice, and the differences in specific activities were less than 10 %.

Cloning of the *dhaA* gene region from *P. pavonaceae* 170

A genomic library of *P. pavonaceae* 170 was constructed by using cosmid vector pLAFR3 according to a previously described procedure (39). Individual cosmid clones were screened for dehalogenase activity by monitoring halide production upon incubation with 1,2-dibromoethane. Out of 5,000 *E. coli* HB101 clones tested, two dehalogenase-positive clones were found. Recombinant cosmids pPC8 and pPC33 encoding haloalkane dehalogenase were isolated from these two HB101 clones and were digested with *Bam*HI. Both cosmids had a 3-kb *Bam*HI fragment in common. The 3-kb *Bam*HI fragment of pPC33 was ligated into the *Bam*HI site of pBluescript SK(-). The ligation mixture was used to transform cells of *E. coli* JM101, and transformants were plated on LBZ plates containing ampicillin, X-gal, and IPTG. Ampicillin-resistant white colonies displaying haloalkane dehalogenase activity with 1,2-dibromoethane were selected. Plasmid DNA (pSK45) of one of these colonies was isolated and used as template for DNA sequencing. The nucleotide sequences of DNA regions flanking the 3-kb *Bam*HI fragment were determined by using cosmid pPC33 directly as a template for DNA sequencing.

Nucleotide sequencing and analysis

DNA sequencing was performed as previously described (21, 36). The nucleotide sequence data were analyzed by using the programs supplied in the DNASTAR software package (DNASTAR Inc., Madison, Wis.) or those supplied in the PC/GENE software package (Genofit, Geneva, Switzerland). Searches for nucleotide and amino acid sequence similarities were carried out by using the BLAST program (3) and the DDBJ, EMBL, and GenBank databases. Protein sequences were aligned by using CLUSTAL W (41), and alignments of nucleotide sequences were made by using LALIGN (Institut de Génétique Humaine, Montpellier, France).

Nucleotide sequence accession numbers

The nucleotide sequence data of the haloalkane dehalogenase gene regions from *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 have been submitted to the DDBJ, EMBL, and GenBank databases under accession no. L49435, AJ250371, and AJ250372, respectively.

RESULTS AND DISCUSSION

Sequence analysis of the *dhaA* gene region from *R. rhodochrous* NCIMB13064

The *dhaA* gene of the 1-chlorobutane-degrading bacterium *R. rhodochrous* NCIMB13064 is located on the 100-kb plasmid pRTL1 (23). A 13.1-kb DNA fragment of pRTL1, including a 8.2-kb region upstream and a 4-kb region downstream of *dhaA*, was sequenced (Fig. 2). The *dhaA* gene sequence and the analysis of insertion element IS*2112*, which is located approximately 6 kb upstream of *dhaA* (Fig. 2), were described previously (21, 23).

Two complete open reading frames (ORFs), designated *invA* and *dhaR*, were found upstream of *dhaA* (Fig. 2 and 3). InvA shares extensive similarity with proteins belonging to the invertase family of site-specific recombinases. The inversion reaction is a site-specific recombination between inverted repeat sequences which flank the invertable DNA fragment and is carried out by invertases. Several examples of invertable DNA that can serve as a genetic switch between the expression of alternative sets of genes have been described (14). InvA is most similar to the invertases Pin of *E. coli* (34) and Hin of *Salmonella enterica* serovar Typhimurium (51) (Table 1). The high amino acid sequence identity with these DNA invertases implies a common phylogenetic origin, although invertase action has yet to be demonstrated for InvA.

Database searches with the deduced amino acid sequence of the *dhaR* gene revealed no proteins with significant similarity to the entire sequence of the *dhaR* product. However,

DhaR contains a region near the N terminus which resembles the helix-turn-helix (HTH) DNA binding motifs of a number of transcriptional regulators (10, 30). Considerable similarity was found with the HTH motifs of the *Streptomyces* autoregulator receptors ArpA, BarA, and FarA (Fig. 4), which were proposed to act as repressor-type regulators for secondary metabolism and morphogenesis (28, 29, 45). The overall sequence similarity to these three *Streptomyces* regulators is low (Table 1).

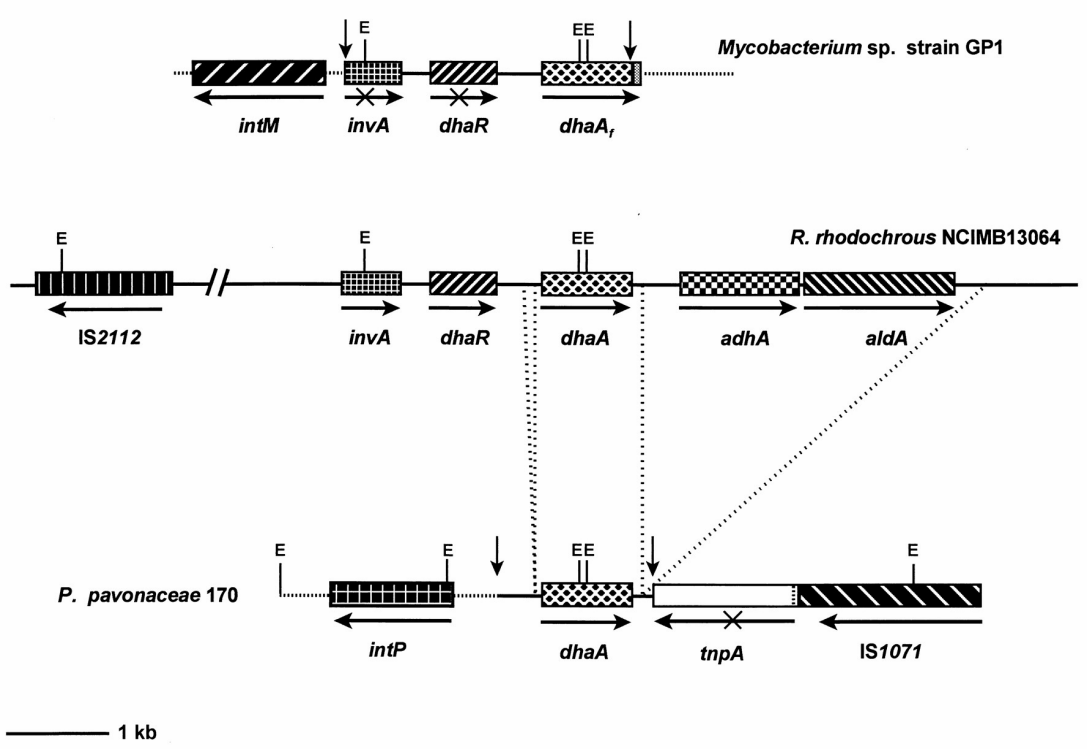

Figure 2. Organization of the haloalkane dehalogenase gene regions of *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1. Genes are shown as hatched boxes, and arrows indicate the direction of transcription. Identical hatching indicates identical genes. Incomplete ORFs are indicated by a cross through the arrow. The borders of the conserved DNA segments in strains 170 and GP1, which are highly similar to segments of the 1-chlorobutane-catabolic gene cluster of strain NCIMB13064, are indicated by vertical arrows. The two deletions within the conserved region of strain 170 are indicated by vertical dotted lines. The 42-nucleotide extension of the *dhaA* ORF in strain GP1, which is the result of a fusion between the *dhaA* segment and haloalcohol dehalogenase HheB encoding DNA (36), is indicated by a shaded box. *Eco*RI restriction sites (E) are shown.

Cells of strain NCIMB13064 grown in *n*-butanol do not possess DhaA activity, but growth on 1-chlorobutane induces the expression of the dehalogenase DhaA (9). The presence of a region resembling the HTH DNA binding motifs in DhaR and the localization of its encoding ORF directly upstream of *dhaA* suggest that DhaR modulates transcription of *dhaA* by binding directly to its promoter region in response to 1-chlorobutane. Sequence analysis of

the *dhaR-dhaA* intergenic region revealed the presence of two identical directly repeated sequences of 13 bp (Fig. 3). The repeated sequences contain a 10-bp palindromic sequence (TGACCGGTCA) and are both part of a larger (imperfect) palindrome. The same 13-bp repeated sequence was also found upstream of *dhaR* (Fig. 3). The presence of these putative binding sites for DhaR upstream of both the *dhaR* and *dhaA* genes suggests that this protein regulates its own expression and that of DhaA. However, promoter sequences for *Rhodococcus* genes are not well characterized (24), and the promoter responsible for *dhaA* expression has not been identified.

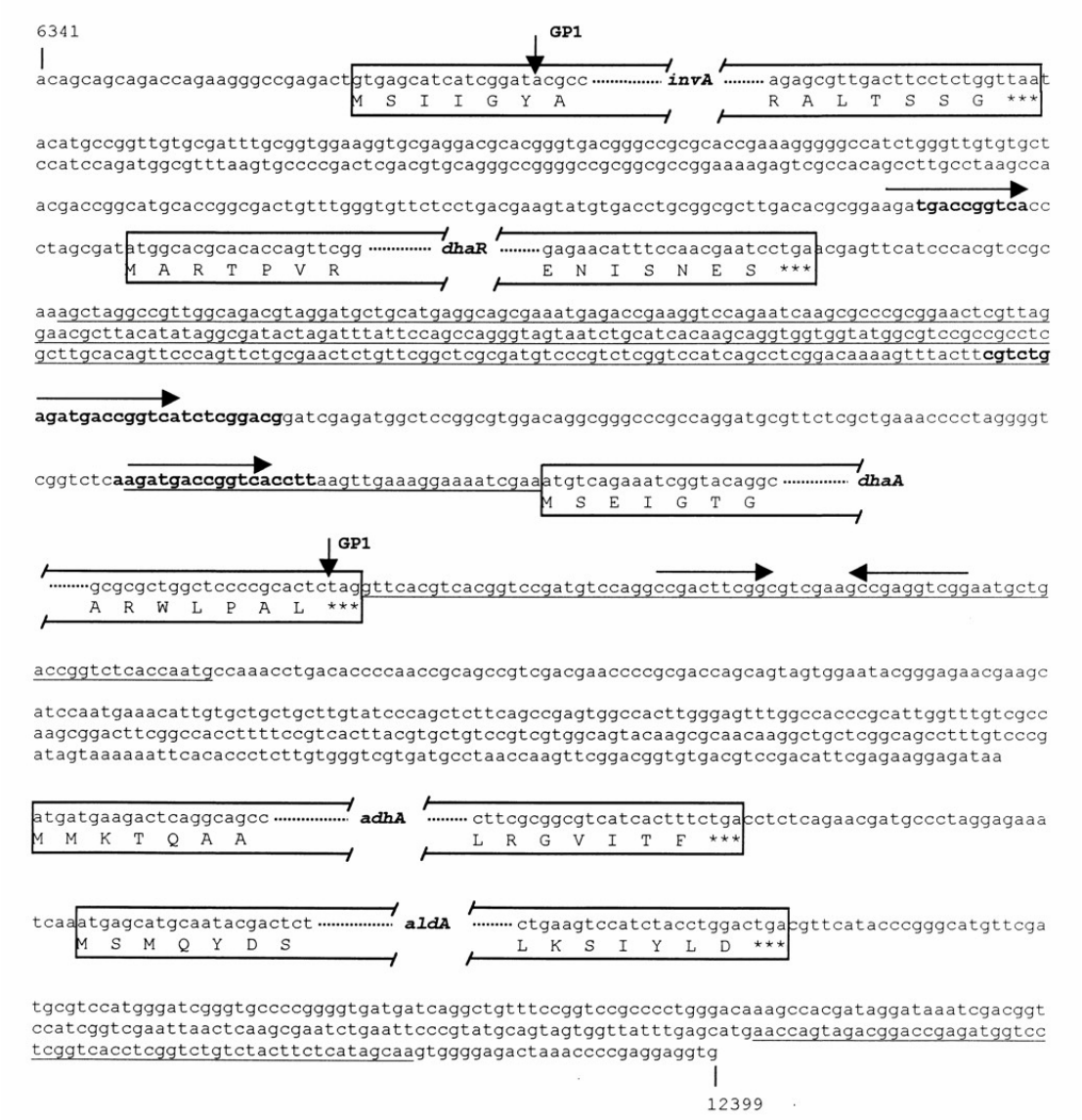

Figure 3. Partial nucleotide sequence of the 1-chlorobutane-degradative gene cluster found in pRTL1 of *R. rhodochrous* NCIMB13064. The borders of the conserved DNA segment in *Mycobacterium* sp. strain GP1 are indicated by vertical arrows. DNA stretches identical to the nucleotide sequences flanking the *dhaA* gene in *P. pavonaceae* 170 are underlined. Inverted and directed repeats are indicated by horizontal arrows above the sequence. Palindromic sequences are shown in boldface.

Table 1. Localization of genes, the corresponding gene products, and identities with other proteins

Strain	Gene	Region of nucleotide sequence[a]	Gene product	Deduced mol wt (kDa)	% Identity with other proteins (references)
NCIMB13064	*invA*	6369-6929	Putative invertase	20.6	Pin, 50 (34); Hin, 48 (51)
	dhaR	7209-7815	Repressor-type regulator	22.7	ArpA, 20 (29); BarA, 20 (28); FarA, 20 (45)
	dhaA	8244-9125	Haloalkane dehalogenase	33.2	See reference 23
	adhA	9543-10655	Putative alcohol dehydrogenase	38.8	AdhD, 50 (8); AdhB, 48 (8); AdhI, 34 (33); AdhS, 33 (32)
	aldA	10687-12135	Putative aldehyde dehydrogenase	50.7	Rv0223C, 47 (8); CymC, 38 (11); DhaL, 38 (46); AldA, 35 (8)
170	*intP*	587-1726[b]	Putative integrase	40.4	See Fig. 6A
	dhaA	2641-3522	Haloalkane dehalogenase	33.2	See reference 23
	*tnpA*Δ	3879-5024[b]	Disrupted transposase	-	TnpA, 51[c]
	tnpA of IS*1071*	5355 > 6900[b]	Transposase	108.4	See reference 26
GP1	*intM*	82-1437[b]	Putative integrase	49.1	See Fig. 6B
	$dhaA_f$	3482-4405	Haloalkane dehalogenase	34.7	See reference 36

[a]The nucleotide sequence numbers refer to the sequences of the haloalkane dehalogenase gene regions of strains NCIMB13064, 170, and GP1 that have been deposited at the DDBJ, EMBL, and GenBank databases under accession no. L49435, AJ250371, and AJ250372, respectively.

[b]Located on the complementary DNA strand when compared to the haloalkane dehalogenase gene.

[c]Accession no. AF028594.

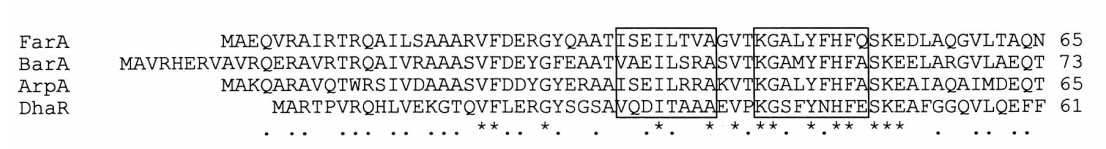

Figure 4. Alignment of the N-terminal amino acid sequences of FarA, BarA, ArpA, and DhaR. Amino acids conserved in all four proteins are indicated by asterisks, whereas amino acids conserved in three out of four proteins are indicated by dots. The sequences corresponding to helix 2 and helix 3 in the HTH DNA binding motif are boxed.

Two ORFs, designated *adhA* and *aldA*, were found downstream of *dhaA* (Fig. 2 and 3). AdhA shares considerable similarity with several proteins that belong to the alcohol dehydrogenase subgroup which contains the NAD(P)- and zinc-dependent long chain alcohol dehydrogenases (Table 1). The highest similarity was found with putative alcohol dehydrogenases from *Mycobacterium tuberculosis* H37Rv (Table 1). Structural and catalytic residues that are conserved among almost all microbial alcohol dehydrogenases (37) are also present in AdhA. AldA shares considerable similarity with putative aldehyde dehydrogenases from *M. tuberculosis* H37Rv and with several known NAD(P)-dependent aldehyde dehydrogenases (Table 1). The cysteine residue (C302, family position) that is strictly conserved among all available sequences of the aldehyde dehydrogenase superfamily, and is proposed to be the active site nucleophile (6, 12), is also conserved in AldA.

Curragh and coworkers (9) showed that 1-chlorobutane was metabolized by strain NCIMB13064 via *n*-butanol and *n*-butyric acid (Fig. 1). 1-Chlorobutane is converted to *n*-butanol by DhaA (23). It therefore appears very likely that *adhA* and *aldA*, which are located downstream of *dhaA* (Fig. 2), encode the alcohol and aldehyde dehydrogenases that are involved in the oxidative conversion of *n*-butanol to *n*-butyric acid. The genes for the initial steps in the degradation of 1-chlorobutane thus appear to be located in a cluster on plasmid pRTL1.

The extent of the conserved *dhaA* gene fragments in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1

When the haloalkane dehalogenase gene regions of *P. pavonaceae* 170 and *R. rhodochrous* NCIMB13064 are compared, the conserved DNA fragment in strain 170 can be seen to include a region of about 1.3 kb that harbors little more than the coding region of the *dhaA* gene (Fig. 2). The *dhaA* genes of strains 170 and NCIMB13064 are completely identical. The first 37 nucleotides upstream of the start codon of *dhaA* are also identical in both strains, after which there is a deletion of 98 nucleotides in the *Pseudomonas* sequence when compared to the *Rhodococcus* sequence (Fig. 2 and 3). Upstream of this deletion, the sequences continue to be identical for 268 nucleotides and then abruptly become completely

unrelated. The 98-nucleotide deletion in the *Pseudomonas* sequence includes exactly the DNA sequence between the 13-bp directed repeat as well as one of the repeated sequences itself (Fig. 3). The formation of this deletion may be explained by DNA strand slippage, which allows one repeated sequence to mispair with the complement of the other (2).

The two sequences are identical for only 77 nucleotides downstream of the *dhaA* gene. The following 60 nucleotides in the *Pseudomonas* sequence (nucleotides 3600 to 3659) are identical to a fragment downstream of *aldA* (nucleotides 12313 to 12372) in the *Rhodococcus* sequence (Fig. 3). This suggests that a large deletion of about 3.2 kb, including the alcohol and aldehyde dehydrogenase genes, has occurred in the *Pseudomonas* sequence when compared to the *Rhodococcus* sequence (Fig. 2). No similarity between the two sequences was found further downstream.

When the haloalkane dehalogenase gene regions of *Mycobacterium* sp. strain GP1 and *R. rhodochrous* NCIMB13064 are compared, the conserved DNA fragment in strain GP1 appears to include a region of about 2.7 kb which harbors *dhaA*, *dhaR*, and part of *invA* (Fig. 2). The similarity of the dehalogenase gene region of strain GP1 to that of strain NCIMB13064 starts at nucleotide 17 of the *invA* ORF and ends exactly before the stop codon of the *dhaA* ORF (Fig. 3). This DNA segment in strain GP1 is identical to the corresponding segment in strain NCIMB13064, except for three nucleotide substitutions in *dhaA* and a 12-nucleotide deletion in the *Rhodococcus* regulatory gene *dhaR*. No further similarity was found between the two sequences.

The haloalkane dehalogenase gene regions of the three phylogenetically different bacteria *R. rhodochrous* NCIMB13064, *P. pavonaceae* 170, and *Mycobacterium* sp. strain GP1 thus appear to have a common genetic origin. The globally distributed 1-chlorobutane catabolic gene cluster on plasmid pRTL1 (*Chapter 5*) seems to be ancestral to the DNA fragments harboring the *dhaA* gene in strains 170 and GP1. These *dhaA* gene fragments probably originated from a catabolic gene cluster similar to the one present on pRTL1 in strain NCIMB13064 and were horizontally transferred to the present hosts. Since the structural genes are identical, we conclude that horizontal transfer of the *dhaA* gene fragment to strain 170 has occurred naturally and recently, resulting in the formation of a newly evolved pathway for 1,3-dichloropropene-degradation (Fig. 1). The hydrolytic product of 1,3-dichloropropene, 3-chloroallyl alcohol, can be used as a carbon source by several gram-negative bacteria (5, 43). We also propose that the 1,2-dibromoethane-degrading pathway of strain GP1 arose by horizontal transfer of a DNA fragment harboring *dhaA* from a donor strain present in the original mixed enrichment culture to a 2-bromoethanol-degrading host. The host that obtained a complete degradation pathway for 1,2-dibromoethane in this way gained a selective advantage and became the predominant organism in the mixed culture.

The role of the *dhaR* gene in the regulation of *dhaA* expression

The sequence analysis indicated that expression of the *dhaA* gene in *R. rhodochrous* NCIMB13064 may be regulated by the *dhaR* gene product. If *dhaR* encodes a regulatory protein, its inactivation should lead to either constitutive expression or noninducibility of the *dhaA* gene, depending on the negative or positive nature of the regulation, respectively. *E. coli* HB101 harboring recombinant cosmid pLTL1k, which carried intact *dhaR* and *dhaA* genes (Fig. 5), constitutively expressed *dhaA* (Table 2), suggesting that expression of *dhaA* is not regulated in *E. coli*. Introduction of pLTL1k into *Pseudomonas* sp. strain GJ1 did not lead to constitutive expression of *dhaA*. We therefore used strain GJ1 to analyze the role of the *dhaR* gene in the regulation of *dhaA* expression.

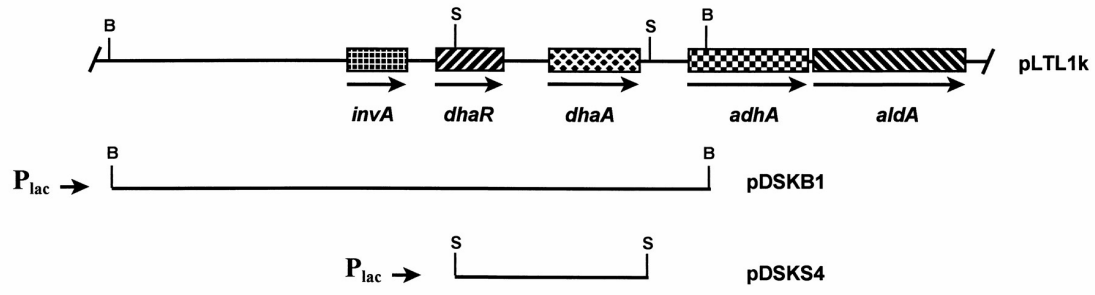

Figure 5. Schematic overview of the 1-chlorobutane-degradative gene cluster in recombinant cosmid pLTL1k and of the two subclones used for *dhaA* expression studies in *Pseudomonas* sp. strain GJ1. Genes are indicated by hatched boxes and arrows indicate the direction of transcription. Cosmid pLTL1k was cut at the indicated restriction sites and the corresponding *dhaA* gene fragments were isolated and inserted into the *Bam*HI or *Sal*I site of pDSK519, yielding plasmids pDSKB1 and pDSKS4, respectively. The direction of the pDSK519-localized *lac* promoter is indicated. *Bam*HI and *Sal*I restriction sites are indicated by B and S, respectively.

Plasmids that carried either *dhaA* and an intact *dhaR* gene (pDSKB1) or *dhaA* and the *dhaR* gene with a deletion in its proximal part (pDSKS4) were constructed (Fig. 5). In contrast to cell extracts prepared from strains GJ1(pLTL1k) and GJ1(pDSKB1), cell extract from strain GJ1(pDSKS4) displayed haloalkane dehalogenase activity (Table 2). Cell extract from strain GJ1 carrying pDSKS5, in which the *Sal*I insert that is present in pDSKS4 was placed in the direction opposite of that of the *lac* promoter of pDSK519, also displayed dehalogenase activity (Table 2), indicating that the constitutive expression of *dhaA* was controlled by its own promoter and not by the *lac* promoter of pDSK519. These results show that inactivation of *dhaR* leads to constitutive expression of the *dhaA* gene, indicating that the *dhaR* gene product putatively acts as a repressor of *dhaA* expression.

In contrast to the negatively regulated expression of *dhaA* in *R. rhodochrous* NCIMB13064, the haloalkane dehalogenase genes in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1 are constitutively expressed (35, 36). The constitutive expression of *dhaA* in

strain 170 may be caused by the absence of a *dhaR* gene. Although strain GP1 possesses the *dhaR* gene in front of $dhaA_f$, the 12-nucleotide deletion present in *dhaR* may inactivate the regulatory protein (DhaR), leading to constitutive expression. To confirm that this deletion in *dhaR* inactivated its gene product, recombinant cosmid pGP1-4B5, which carried the $dhaA_f$ gene region of strain GP1 (Fig. 2), was introduced into *Pseudomonas* sp. strain GJ1. In contrast to cell extract prepared from strain GJ1 harboring cosmid pLTL1k, which carried the *dhaA* gene region of strain NCIMB13064 (Fig. 5), cell extract of strain GJ1(pGP1-4B5) displayed haloalkane dehalogenase activity (Table 2), indicating that the deletion in *dhaR* had inactivated its gene product. This further emphasizes the negative regulatory role of DhaR.

Table 2. Dehalogenase activities in crude extracts of *E. coli* HB101 and *Pseudomonas* sp. strain GJ1 harboring different constructs

Strain	Dehalogenase sp act (mU/mg of protein)[a]
HB101 (pLTL1k)	120
GJ1 (pLTL1k)	<20
GJ1 (pDSKB1)	<20
GJ1 (pDSKS4)	1400
GJ1 (pDSKS5)	1380
GJ1 (pGP1-4B5)	1330

[a] Specific activities with 1,2-dibromoethane (5 mM) were determined with extracts prepared from Luria Broth-grown cells.

In strains 170 and GP1, a putative DNA integrase gene is present next to the conserved *dhaA* gene fragment

The sequence comparisons indicated that novel catabolic pathways for 1,3-dichloropropene and 1,2-dibromoethane were built by adding existing DNA fragments harboring *dhaA* to the genomes of *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1, respectively. This suggests the involvement of a mechanism for the acquisition of distinct DNA fragments. Gene acquisition by bacterial genomes could occur either by excisive-integrative recombination events mediated by insertion elements or by site-specific integration events mediated by DNA integrases (40, 42, 47).

Interestingly, an ORF encoding a putative DNA integrase (*intP*) was found upstream of the conserved *dhaA* gene fragment in strain 170 (Fig. 2 and Table 1). IntP shares significant similarity with proteins belonging to the integrase (Int) family of site-specific recombinases (Fig. 6A). Although the members of the Int family exhibit a large diversity in their sequences, they all harbor two regions of marked sequence similarity, called box I and box II (27). IntP

Figure 6. (A) Amino acid sequence alignment of IntP with the conserved segments of the recombinases Cre of bacteriophage P1 (accession no. P06956) and XerD of *E. coli* (M54884) and the integrase-like proteins of *S. ruminantium* (AB011029) and Tn*5041* (X98999). Asterisks represent bases identical to those in the upper sequence. Invariant amino acid residues which are believed to be involved in catalysis are numbered (IntP numbering) and shown in boldface. (B) Amino acid sequence alignment of IntM with the C termini of the recombinases Sss of *P. aeruginosa* (S61402), XerC of *E. coli* (P22885), and CodV of *Bacillus subtilis* (P39776) and the putative phage type integrase of *Sphingomonas aromaticivorans* (AF079317). Asterisks represent bases identical to those in the upper sequence. Dashes represent bases absent in other sequences. The conserved residues His-352, Arg-355, and Tyr-415 are numbered (IntM numbering) and shown in boldface.

shows 55 % identity to its nearest neighbor, the integrase-like protein of *Selenomonas ruminantium*, in these two conserved domains (Fig. 6A). The tetrad R-H-R-Y, which includes the active-site tyrosine (31), is conserved in almost all members of the Int family (1, 4, 27) and is present as Arg-183, His-327, Arg-330, and Tyr-362 in IntP.

An ORF encoding a putative DNA integrase (*intM*) was also found upstream of the conserved *dhaA* gene fragment in strain GP1 (Fig. 2 and Table 1). The C-terminal region of IntM shares considerable similarity with proteins of the Int family (Fig. 6B). The conserved tetrad R-H-R-Y of the Int family (1, 4, 27) is present as Arg-260, His-352, Arg-355, and Tyr-415 in IntM (Fig. 6B).

The presence of an integrase gene in the vicinity of the conserved *dhaA* gene fragment both in strain 170 and in strain GP1 suggests acquisition of these DNA fragments by site-specific integration. Assuming that no additional recombinations have taken place after the primary insertion event, comparisons of the boundaries between the conserved *dhaA* gene fragments and the sequences unique for strain 170 or GP1 define the insertion site. No short duplication of bases on either site of the presumed point of insertion or regions of dyad symmetry that could serve as integrase binding sites were found when the flanking sequences

were compared. The absence of these features could be due to an adjacent deletion removing the border segment on one side. Such a deletion could also explain the fusion of the *dhaA* gene to the 3' end of the haloalcohol dehalogenase gene *hheB* (Fig. 2).

The presence of insertion elements in the vicinity of the conserved *dhaA* gene fragments

Many catabolic genes are associated with transposons (42, 47), and transposition is clearly a major mechanism for the acquisition of catabolic genes by bacterial genomes. Sequence analysis of the regions (> 4 kb) adjacent to the conserved *dhaA* gene fragment in *Mycobacterium* sp. strain GP1 showed that these regions do not encode any proteins related to known transposases. Distal to the *dhaA* gene fragment in *P. pavonaceae* 170, however, a large ORF (*tnpA*) was found (Fig. 2) of which the deduced amino acid sequence showed extensive similarity with the putative transposase of *Pseudomonas pseudoalcaligenes* JS45 (Table 1). The *tnpA* ORF, however, appeared to be interrupted by an insertion element, identified as IS*1071* (Fig. 2), causing a truncation of its product. Nucleotide sequencing of a 1.7-kb fragment of IS*1071* revealed no differences with the known sequence of IS*1071*, which is involved in transposition of the chlorobenzoate genes (26). PCR amplification, using a primer specific for both ends of IS*1071* (due to the inverted repeats at its termini) and recombinant cosmid pPC33 as template DNA, showed the formation of a 3.2-kb PCR product corresponding to the size of IS*1071* (26). Thus, a complete copy of IS*1071* was present as an insertion in the *tnpA* gene approximately 1.6 kb downstream of *dhaA*.

Southern hybridization analysis revealed five copies of IS*1071* in strain 170 but only two copies in strain 170M4, a spontaneous mutant of strain 170 that has lost the *dhaA* gene (35) (results not shown). The 60-kb plasmid pPC170, that was previously identified in strain 170 (44), is still present in strain 170M4, showing that the *dhaA* gene is not plasmid localized. Assuming that the mutation in strain 170M4 was caused by a single deletion event, this could suggest that the *dhaA* gene region in strain 170 is flanked by several copies of IS*1071*, forming a composite class I element, and that a homologous recombination event between two insertion sequences was responsible for the loss of the *dhaA* gene and three copies of IS*1071*. Strain 170M4 also lacked the integrase gene *intP*, which is consistent with such a deletion event.

Many antibiotic resistance genes found on transposons in gram-negative bacteria are located within a conserved DNA sequence (15, 25, 40). These conserved elements, called integrons (16, 40), are formally distinct from other genetic elements in that they determine site-specific integration functions, a DNA integrase and a recombination site, and are thus able to acquire resistance genes at the specific site without the need for the presence of insertion sequences or integrase genes in the DNA segments that are acquired. If the integrase gene *intP* in *P. pavonaceae* 170 is flanked by IS*1071* sequences and confers on this transposon a capability of taking up individual and unrelated catabolic genes by integrase-mediated recombination, strain 170 may possess a mobile DNA element similar to the

previously identified integrons which are capable of taking up antibiotic resistance genes (16, 40).

No sequences similar to IS*1071* were found in strains NCIMB13064 and GP1, indicating that this insertion element was not involved in distribution of the *dhaA* gene among these strains. Similarly, insertion sequence IS*2112*, which is located approximately 6 kb upstream of the *dhaA* gene in strain NCIMB13064 (Fig. 2) (21), is not present in strains 170 and GP1. These results are consistent with the hypothesis that site-specific integration events mediated by IntP and IntM were responsible for the acquisition of the *dhaA* gene fragments, rather than excisive-integrative recombination events mediated by insertion elements. However, the presence of multiple copies of an active mobile element (IS*1071*) around the *dhaA* gene in strain 170, combined with the fact that strain 170 was recently isolated from a 1,3-dichloropropene contaminated environment in which natural genetic exchange is likely to be important (44), suggests that transposition has played a part in the mobilization of the *dhaA* gene among members of the microbial community present in this environment.

Concluding remarks

Our data provide further support for previous studies suggesting that horizontal transfer of genes involved in pollutant biodegradation may play an important role in the evolution of catabolic pathways and the adaptation of microbial communities to different environmental contaminants. Up to now, catabolic genes were thought to be transferred mainly by means of conjugative plasmids and transposons (42, 47). The results presented here suggest that genes specifying adaptation to xenobiotics can also spread as integrons, as has been proposed in the case of genes specifying antibiotic resistance (16, 40). The widespread use of antibiotics and the introduction of xenobiotics into the environment seem to lead to adaptation by similar molecular mechanisms.

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Chapter 5

Haloalkane-utilizing *Rhodococcus* strains isolated from geographically distinct locations possess a highly conserved gene cluster encoding haloalkane catabolism

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The sequences of the 16S rRNA and haloalkane dehalogenase (*dhaA*) genes of five gram-positive haloalkane-utilizing bacteria isolated from contaminated sites in Europe, Japan, and the United States and of the archetypal haloalkane-degrading bacterium *Rhodococcus* sp. strain NCIMB13064 were compared. The 16S rRNA gene sequences showed less than 1% sequence divergence and all haloalkane degraders clearly belonged to the genus *Rhodococcus*. All strains shared a completely conserved *dhaA* gene, suggesting that the *dhaA* genes were recently derived from a common ancestor. The genetic organization of the *dhaA* gene region in each of the haloalkane degraders was examined by hybridization analysis and DNA sequencing. Three different groups could be defined on the basis of the extent of the conserved *dhaA* segment. The minimal structure present in all strains consisted of a conserved region of 12.5 kb, which included the haloalkane-degradative gene cluster that was previously found in strain NCIMB13064. Plasmids of different sizes were found in all strains. Southern hybridization analysis with a *dhaA* gene probe suggested that all haloalkane degraders carry the *dhaA* gene region both on the chromosome and on a plasmid (70 to 100 kb). This suggests that an ancestral plasmid was transferred between these *Rhodococcus* strains and subsequently has undergone insertions or deletions. In addition, transposition events and/or plasmid integration may be responsible for positioning the *dhaA* gene region on the chromosome. The data suggest that the haloalkane dehalogenase gene regions of these gram-positive haloalkane-utilizing bacteria are composed of a single catabolic gene cluster that was recently distributed worldwide.

INTRODUCTION

Hydrolytic dehalogenation by haloalkane dehalogenases is the most important step in the biodegradation of 1-halo-*n*-alkanes and α,ω-dihalo-*n*-alkanes. Haloalkane dehalogenases active against long-chain haloaliphatic compounds are present in the gram-positive haloalkane-utilizing bacterial strains Y2, isolated in the United Kingdom (16), m15-3, isolated in Japan (25), HA1, isolated in Switzerland (20), GJ70, isolated in The Netherlands (7), and NCIMB13064, isolated in the United Kingdom (2). These enzymes are different from the extensively studied haloalkane dehalogenase (DhlA) found in several gram-negative 1,2-dichloroethane-utilizing bacteria of the genera *Xanthobacter* and *Ancylobacter* (8, 23). DhlA is able to dehalogenate 1,2-dichloroethane, which is a poor substrate for other haloalkane dehalogenases, whereas the enzymes isolated from the gram-positive strains exhibit higher activity toward long-chain mono- and dihalogenated substrates.

Rhodococcus sp. strain NCIMB13064 has been used to study the genetics of haloalkane metabolism in gram-positive bacteria. It is host to the self-transmissible 100-kb plasmid pRTL1 (11), which carries the genes (*dhaA*, *adhA* and *aldA*) for the initial steps in the aerobic degradation of 1-chlorobutane and several other 1-halo-*n*-alkanes (Fig. 1). The *dhaA* gene codes for a haloalkane dehalogenase that hydrolytically converts 1-chlorobutane to *n*-butanol (12). Based on amino acid sequence similarities, the *adhA* and *aldA* genes, which are located downstream of *dhaA* (Fig. 1B), were proposed to encode the alcohol and aldehyde dehydrogenases that catalyze the oxidative conversion of *n*-butanol to *n*-butyric acid (15a). The latter compound is further metabolized by β-oxidation to acetate, which can serve as a growth substrate for many bacteria (2). The 13-kb DNA fragment of pRTL1 that has been sequenced harbors two additional genes, *dhaR* and *invA*, and the insertion sequence IS*2112* (Fig. 1B). The *dhaR* gene product putatively acts as a repressor-type regulator for *dhaA* expression (15a), and IS*2112* was shown to be involved in several genome rearrangements that resulted in the loss of haloalkane dehalogenase activity in strain NCIMB13064 (10). The *invA* gene encodes a putative protein that shares extensive similarity with the DNA invertase Pin of *Escherichia coli* (50%) and Hin of *Salmonella enterica* serovar Typhimurium (48%) (15a). The Pin and Hin invertases are responsible for the inversion of a specific DNA fragment that can serve as a genetic switch between the expression of alternative sets of genes (4). However, it is not known whether the putative invertase gene *invA* plays an analogous role in regulating haloalkane metabolism in *Rhodococcus* sp. strain NCIMB13064.

Although the haloalkane dehalogenases of strains Y2, m15-3, HA1, and GJ70 have been characterized (7, 16, 20, 25), nothing is known about the genetics of haloalkane degradation in these strains. The biochemical characteristics of the haloalkane dehalogenases isolated from these strains, however, closely resemble those of the haloalkane dehalogenase (DhaA) from *Rhodococcus* sp. strain NCIMB13064. Furthermore, the amino-terminal

sequences of the dehalogenases from strains Y2 and HA1 are identical to that of DhaA. This apparent homology raises interesting questions about the evolution and distribution of these enzymes since the organisms were isolated from geographically separate locations. To determine the degree of homology between the haloalkane dehalogenases and to obtain insight in their evolution and distribution, we analyzed the genetic organization and genomic location of the haloalkane dehalogenase gene region in each of the strains Y2, m15-3, HA1, and GJ70 and in another gram-positive haloalkane-degrading bacterium, strain TB2, newly isolated from an industrial site in the United States. In addition, we determined the phylogenetic affiliation of the different haloalkane degraders by 16S rRNA gene sequence analysis. The results indicate that the analyzed gram-positive haloalkane degraders are members of the genus *Rhodococcus*, and despite the fact that they were isolated independently from different continents, they all possess a haloalkane catabolic gene cluster highly similar to the one found on plasmid pRTL1 in *Rhodococcus* sp. strain NCIMB13064.

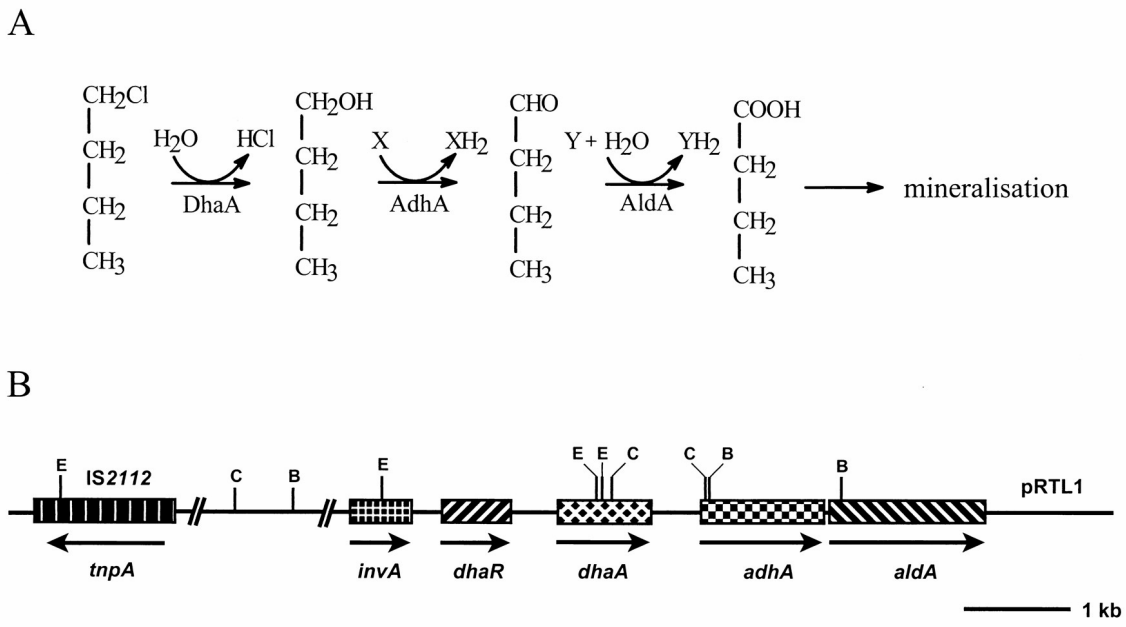

Figure 1. (A) Initial enzymatic steps in the degradation of 1-chlorobutane by *Rhodococcus* sp. strain NCIMB13064. Below each conversion step, the responsible enzyme is indicated (DhaA, haloalkane dehalogenase; AdhA, alcohol dehydrogenase; AldA, aldehyde dehydrogenase). (B) Genetic organization of the haloalkane-degradative genes *dhaA*, *adhA*, and *aldA*, the regulatory gene *dhaR*, the putative invertase gene *invA*, and insertion element IS*2112* on plasmid pRTL1 in *Rhodococcus* sp. strain NCIMB13064 (as determined by DNA sequencing [15a]; accession no. L49435). Arrows indicate the direction of transcription. Solid line, noncoding pRTL1 DNA. The positions of *Bam*HI, *Cla*I, and *Eco*RI restriction sites are important for explaining the hybridization results obtained in this study and are indicated by B, C, and E, respectively.

MATERIALS AND METHODS

Haloalkane-utilizing bacteria used in this study

The bacterial strains screened for the presence of the *dhaA* gene are listed in Table 1. Strains Y2, m15-3, HA1, and GJ70 were isolated from widely separated sites by different research groups (6, 16, 19, 24). Strains NCIMB13064 and 170 (formerly known as *Pseudomonas cichorii* 170) have been investigated previously and were shown to possess identical *dhaA* genes (12, 14). Strain TB2 was isolated in our laboratory from a soil sample obtained from a trichloropropane-polluted site in the United States and is capable of growth with 1-chlorobutane as the sole carbon and energy source.

Table 1. List of haloalkane-utilizing strains carrying the *dhaA* gene

Strain[a]	Origin[b]	Reference(s) or source
Strain Y2	UK; industrial site exposed to haloalkanes	16
Strain NCIMB13064	UK; industrial site exposed to chlorinated alkanes	2, 12
Strain m15-3	Japan	24, 25
Strain HA1	Switzerland	19, 20
Strain GJ70	The Netherlands; sludge contaminated with pesticides	6, 7
Strain TB2	USA; industrial site exposed to 1,2,3-trichloropropane	This study
P. pavonaceae 170	The Netherlands; soil exposed to 1,3-dichloropropene	14

[a] On the basis of the 16S rRNA gene sequence analysis performed in this study, strains Y2, NCIMB13064, m15-3, HA1, GJ70, and TB2 should be classified as strains of *Rhodococcus erythropolis*.
[b]UK, United Kingdom; USA, United States.

PCR amplification, cloning, and sequencing of putative *dhaA* genes

Total genomic DNA was isolated from nutrient broth-grown cells by a phenol extraction procedure described elsewhere (14) and was directly used as the template for PCR amplification. Putative *dhaA* genes were amplified with the same primers that were successfully used to amplify the *dhaA* gene from *Pseudomonas pavonaceae* 170 (14): 5'-AAAATCGCCATGGCAGAAATCGGTA-3' and 5'-TGGACATCGGACCATGGCGTGA-ACC-3' (*Nco*I sites are underlined). The amplification reaction mixture (100 μl) contained standard *Taq* amplification buffer, 250 μM (each) deoxyribonucleotide triphosphate, 100 ng of each primer, 100 ng of genomic DNA, and 2 U of *Taq* DNA polymerase (Boehringer GmbH, Mannheim, Germany). The cycling parameters were 94ºC for 5 min followed by 30 cycles of 94ºC for 60 s, 58ºC for 60 s, and 72ºC for 90 s, with a final elongation step of 72ºC

for 10 min. Samples (10 μl) of the reaction mixtures were subjected to electrophoresis in 0.8% agarose gels, and PCR products were stained with ethidium bromide.

The PCR-amplified *dhaA* genes of strains GJ70 and Y2 were directly cloned in the TA cloning vector pCR2.1 according to the recommendations of the supplier (Invitrogen, Leek, The Netherlands). PCR products obtained for strains TB2, m15-3, and HA1 were cloned in the *Nco*I site behind the T7 promoter in the expression vector pGEF+ as described before (14). Both DNA strands of the cloned dehalogenase genes were sequenced by the dideoxy chain termination mehod (28), and nucleotide sequences were aligned by using LALIGN (Institut de Génétique Humaine, Montpellier, France).

16S rRNA gene sequence analysis

The 16S rRNA genes were amplified with the oligonucleotide primers that were described by Marchesi et al. (13): 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGG(A/T)GTGTACAAGGC-3') (numbering based on the *E. coli* 16S rRNA gene [1]). The amplification reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.01% bovine serum albumin, 0.01% gelatin, 0.05 % Triton-X-100, 200 μM (each) deoxyribonucleotide triphosphate, 0.5 μM (each) primer, 100 ng of genomic DNA, and 1 to 2.5 U of *Taq* DNA polymerase. The cycling parameters were 94ºC for 2 min followed by 30 cycles of 92ºC for 30 s, 55ºC for 30 s, and 75ºC for 45 s, with a final elongation step of 75ºC for 5 min.

Amplification products were cloned into plasmid pTAg as specified by the manufacturer (R&D Systems, Abingdon, United Kingdom). Cloned 16S rRNA genes were cycle sequenced using the Amersham Thermo Sequenase cycle sequencing kit with 7-deaza-dGTP and IRD-41-labeled primers that annealed to the M13 universal and reverse sites of the pTAg vector. Sequencing reaction mixtures were run on an automatic sequencing machine (Li-Cor 4000L; MWG-Biotech). Both DNA strands were sequenced to ensure accuracy. Phylogenetic analysis on the newly determined 16S rRNA gene sequences was done essentially as decribed before (15).

Plasmid visualization

Rhodococcus strains were grown in 10 ml of yeast extract-peptone medium (11) at 28ºC to an optical density at 600 nm of 0.9 to1.0. Cultures were diluted (1:1) with 0.9% NaCl, after which cells from 2 ml of culture were collected in 1.5-ml tubes. Cell pellets were resuspended in 100 μl of 20% Ficoll (approximate molecular weight, 400,000) in 1 × Tris-acetate (TAE) buffer (17). Resuspended cells were mixed with 5 μl of fresh lysozyme solution (18% Ficoll, 0.18% bromophenolblue, lysozyme [2.4 mg/ml], and RNase [0.24 mg/ml] in TAE) and incubated for 10 min at room temperature. Then, 15 μl of 2% sodium dodecyl sulfate (SDS)-10% Ficoll in TAE was added, and preparations were incubated at 60ºC for 20 to 30 min. The mixtures were subsequently transferred into the wells of a 0.9% vertical

agarose slab, from which the TAE buffer was carefully removed. Mixtures were overlaid with 50 µl of 2% SDS-5% Ficoll in TAE, after which the wells were sealed with melted agarose. Electrophoresis was performed in TAE buffer for 40 min at 1V/cm and then at 5 to 6V/cm until completion. Plasmid DNA was stained with ethidium bromide.

Southern hybridizations

DNA fragments or intact plasmids were separated on an agarose gel and subsequently transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech) or to a positively charged nylon membrane (Boehringer GmbH) by diffusion blotting (17). The membranes were treated according to the instructions of the manufacturer. Parts of the *dhaA* gene or of IS*2112* were amplified by PCR and were purified using the Qiaquick PCR purification kit (Qiagen) or by using the GFX PCR DNA and gel band purification kit (Amersham). These PCR fragments were labeled with digoxigenin-11-dUTP using the nonradioactive DNA labeling and detection kit from Boehringer GmbH or with fluorescein-11-dUTP by using the gene images random prime labeling module of Amersham and were used as hybridization probes. Hybridization was carried out at 62 to 68ºC, and detection of the hybridization signals was performed according to the manufacturer's protocol.

The complete *dhaA* gene was amplified by using the primers and conditions described above. An internal part of the *dhaA* gene (550 bp) was obtained by using the following oligonucleotide primers: f (5'-GACGACCACGTCCGCTACC-3') and r (5'-CCGATGTCCA-CTGTCTTGC-3'). The IS*2112* hybridization probe (nucleotide positions 17 to 1108 of the *tnpA* open reading frame [ORF]) was generated with primers f (5'-CCCACGCATGGGTCG-3') and r (5'-CGACGCGCCAAGGCG-3'). Conditions used for the amplification of IS*2112* have been described previously (10).

Cloning and sequencing of the *dhaA* gene regions of strains GJ70 and HA1

To obtain the *dhaA* gene regions of strains GJ70 and HA1, genomic libraries of these strains were constructed using the cosmid vector pLAFR3 (21). Total DNA was partially digested with *Sau*3A, and fragments of 15 to 25 kb in size were isolated and ligated into the *Bam*HI site of pLAFR3. Ligation mixtures were packaged in vitro by using the DNA packaging kit of Boehringer GmbH. *E. coli* HB101 was transduced with these packaging mixtures (17), and colonies were selected on LB agar plates without NaCl (LBZ) (15) containing tetracycline (12.5 µg/ml). Tetracycline-resistant colonies were screened for dehalogenase activity by monitoring halide production upon incubation with 1,2-dibromoethane as described before (15).

Recombinant cosmids encoding haloalkane dehalogenase activity were isolated and directly used as the template for sequencing DNA regions upstream of the *invA* gene. Oligonucleotide primers were designed on the basis of the known sequence of the *dhaA* gene region of *Rhodococcus* sp. strain NCIMB13064 (accession no. L49435). DNA sequencing

was performed as described elsewhere (15a), and newly determined sequences for strains GJ70 and HA1 were aligned with the known sequence of the *dhaA* gene region of strain NCIMB13064 by using the program LALIGN.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of strains TB2, m15-3, HA1, Y2, NCIMB13064, and GJ70 have been submitted to the DDBJ/EMBL/GenBank databases under accession no. AJ250924, AJ250925, AJ250926, AJ250927, AJ250928, and AJ250929, respectively. The nucleotide sequence data of the regions upstream of the *invA* gene in strains GJ70 and HA1 have been submitted under accession no. AJ250982 and AJ250983, respectively.

RESULTS AND DISCUSSION

16S rRNA gene sequence analysis

The haloalkane-utilizing bacterial strains Y2, NCIMB13064, m15-3, HA1, GJ70, and TB2 were isolated by different research groups from geographically distinct locations (Table 1). The first four strains were previously identified as *Rhodococcus erythropolis* Y2 (16), *R. rhodochrous* NCIMB13064 (2), *Corynebacterium* sp. strain m15-3 (25), and *Arthrobacter* sp. strain HA1 (19). Strain GJ70 was previously identified as a gram-positive actinomycete-like organism (7). In order to establish precisely the phylogenetic affiliation of these haloalkane-utilizing bacteria, we determined their 16S rRNA gene sequences.

A comparison of the newly determined 16S rRNA gene sequences revealed less than 1% sequence divergence and clearly places the six haloalkane degraders within the same genus. Phylogenetic analysis was conducted by comparing the 16S rRNA gene sequences for these strains with other 16S rRNA gene sequences available in the GenBank database and in the Ribosomal Database Project. This analysis revealed that the haloalkane degraders are members of the genus *Rhodococcus* and are all most closely related to *R. erythropolis* (Fig. 2); the pairwise sequence identities were >99%. This high 16S sequence identity indicates that the six haloalkane degraders should be classified as new strains of *R. erythropolis* or a closely related species. The results thus show that strains m15-3 and HA1 were previously identified incorrectly (19, 25) and that, like strains GJ70 and TB2, they should be (re)assigned to the genus *Rhodococcus* (26).

Sequence identity among *dhaA* genes of haloalkane-utilizing bacteria

Haloalkane dehalogenases active against long-chain haloaliphatic compounds were previously isolated from strains Y2 (16), m15-3 (25), HA1 (20), and GJ70 (7). On the basis of their substrate specificities and their identical N-terminal amino acid sequences, these four

dehalogenases have been classified as one group of related enzymes (3). The biochemical characteristics and amino-terminal sequences of these dehalogenases suggest that they closely resemble the haloalkane dehalogenase (DhaA) that had been found in strain NCIMB13064 (12) and recently also in *P. pavonaceae* 170 (14). To determine the extent of sequence similarity between the haloalkane dehalogenase genes of strains Y2, m15-3, HA1, and GJ70 and the *dhaA* genes that are present in strains NCIMB13064 and 170, the putative *dhaA* genes of the first four strains were amplified with primers designed for the 5' and 3' ends of the *dhaA* gene (12). In addition, we also screened the newly isolated strain TB2 for the presence of the *dhaA* gene.

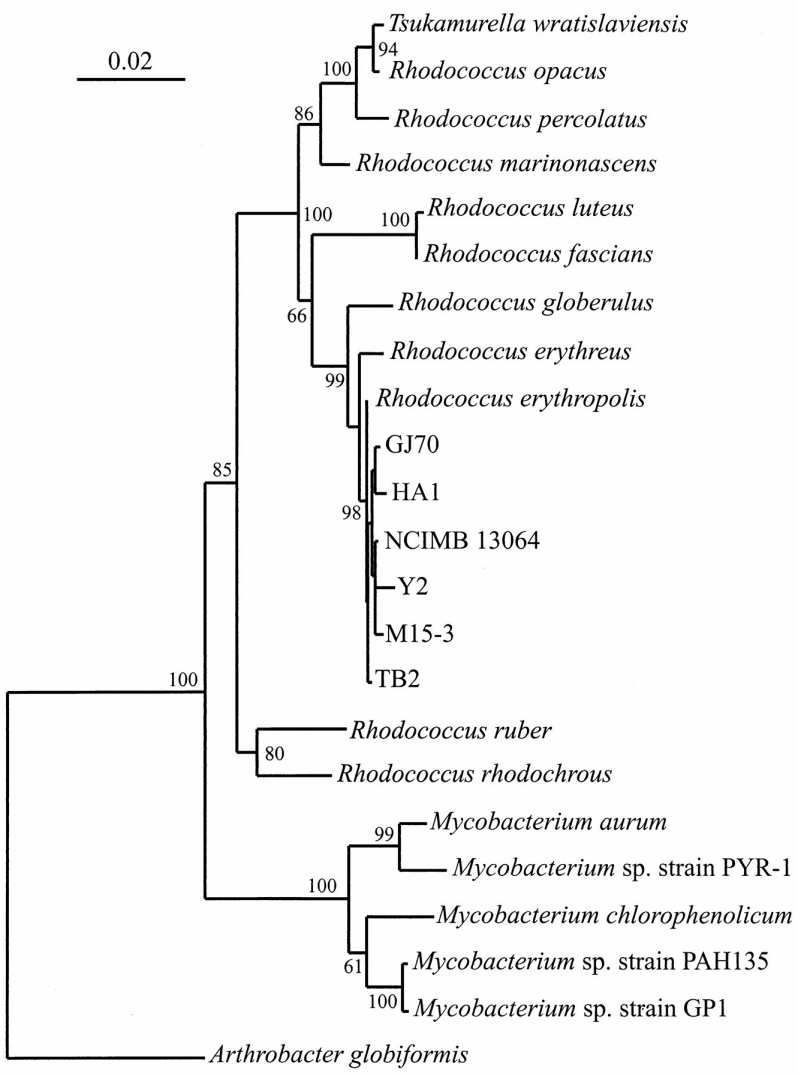

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence analysis, illustrating the relationships of the six haloalkane-degrading strains GJ70, HA1, NCIMB13064, Y2, m15-3, and TB2 to the most closely related bacteria. Base positions 54 to 1368 (numbering based on the *E. coli* 16S rRNA gene) were included in the analysis. Scale bar, 0.02 fixed mutation per site. Bootstrap values were derived from 500 analyses. A sequence from *Arthrobacter globiformis* was used as the outgroup.

For the five strains analyzed here, PCR amplification with *dhaA*-specific primers consistently produced a 0.9-kb DNA fragment corresponding to the size of the *dhaA* gene (data not shown). DNA sequencing revealed that the PCR-amplified haloalkane dehalogenase genes of strains Y2, m15-3, HA1, GJ70, and TB2 were completely identical to the *dhaA* genes of strains NCIMB13064 and 170. Since the lineage of the *dhaA* gene is less diverged (100% sequence indentity) than that of the hosts, as determined by 16S rRNA gene sequence analysis (see above), the *dhaA* gene has probably spread among these bacterial strains by lateral transfer. Thus the haloalkane dehalogenases isolated and characterized from these chloroalkane-utilizing bacteria (2, 7, 14, 16, 25) are all identical to the dehalogenase that was first described in strain m15-3 by Yokota et al. (25).

Genetic organization of the *dhaA* gene regions in haloalkane-utilizing *Rhodococcus* strains

Southern blot hybridization analysis under high-stringency conditions with *dhaA*- and IS*2112*-specific probes was used to determine the extent of the conserved *dhaA* segments in strains m15-3, HA1, Y2, TB2, and GJ70, compared to the sequenced *dhaA* gene region of strain NCIMB13064 (Fig. 1B). When the complete *dhaA* gene was used as the probe, *Bam*HI-digested DNA of strains HA1, m15-3, NCIMB13064, Y2, and TB2 revealed single hybridization signals at about 5.6 kb (data not shown). For *Bam*HI-digested DNA of strain GJ70, a single hybridization signal at about 9 kb was found. We examined the regions flanking the *dhaA* gene by digesting the genomic DNAs with *Eco*RI or *Cla*I. Since the *dhaA* gene has two restriction sites for *Eco*RI close to each other (Fig. 1B), only two hybridizing bands were expected, and these were found. *Eco*RI-digested DNA gave hybridization signals at about 9 kb and 2 kb for all strains (data not shown). The 2-kb DNA band was identical in size to the *Eco*RI restriction fragment that includes the *dhaR* gene in strain NCIMB13064 (Fig. 1B). These results suggest that the six haloalkane degraders possess a conserved DNA segment of at least 11 kb, including a 1.5-kb region upstream, and an 8.5-kb region downstream, of the *dhaA* gene. Since the *dhaA* gene has one cutting site for *Cla*I (Fig. 1B), two DNA bands hybridized with the *dhaA* gene probe (Fig. 3). One fragment (0.9 kb) was found in DNA from all six strains, which is in agreement with a conserved region downstream of the *dhaA* gene (Fig. 1B). The size of the second DNA fragment, however, was different for some strains: 5.4 kb for strains m15-3, NCIMB13064, and TB2; 4 kb for strains HA1 and Y2; and 3.5 kb for strain GJ70. These results imply that the DNA regions upstream of the conserved 11-kb *dhaA* gene segment (upstream of the *Eco*RI site in the *invA* gene) in strains HA1, Y2, and GJ70 are different from those in strains NCIMB13064, m15-3, and TB2.

To compare the DNA regions upstream of the *Eco*RI site in the *invA* gene in strains HA1 and GJ70 in more detail with that in strain NCIMB13064 and to determine whether the conserved DNA fragments in these strains include the entire *invA* gene, we cloned and sequenced the corresponding regions from strains HA1 and GJ70. The NCIMB13064 and

GJ70 sequences were still very similar (>99% sequence identity) for approximately 1.7 kb upstream of the *Eco*RI site in the *invA* gene and then abruptly became completely unrelated (Fig. 4). When the corresponding region of strain HA1 was compared to that of strain NCIMB13064, three conserved DNA segments having up to 35% differences were found. The first approximately 1,000 nucleotides upstream of the *Eco*RI site in the *invA* gene in strain HA1 were still very similar (>99% sequence identity) to the corresponding region in strain NCIMB13064. This segment (Fig. 4) is followed by a segment of approximately 200 nucleotides that shows 35% sequence divergence and a segment of approximately 300 nucleotides that differs from the corresponding segment in strain NCIMB13064 in about 10% of the positions. No further similarity between the two sequences was found. These results show that, compared to the sequenced *dhaA* gene region of strain NCIMB13064, the conserved DNA fragments in strains GJ70 and HA1 extend to approximately 1.7 and 1.5 kb, respectively, beyond the *Eco*RI site in the *invA* gene and thus include the complete *invA* gene. Since the sequence similarity between strains HA1 and NCIMB13064 vanishes before the *Bam*HI site that is located upstream of the *invA* gene (Fig. 4), the localization of the *dhaA* genes in both strains on *Bam*HI restriction fragments of approximately the same size (about 5.6 kb; see above) must be coincidental.

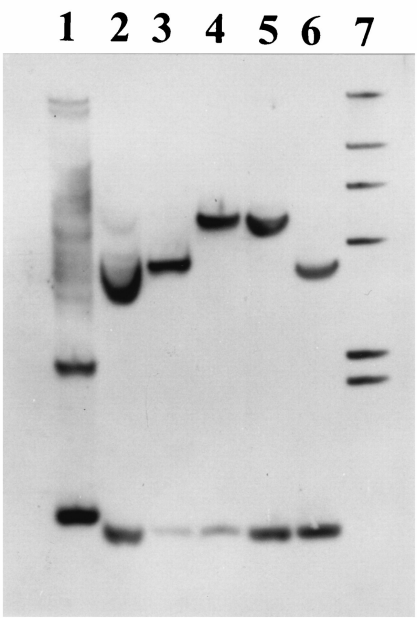

Figure 3. Results of a Southern hybridization experiment with a *dhaA* gene probe and *Cla*I-digested total DNA of strains GJ70 (lane 2), HA1 (lane 3), m15-3 (lane 4), NCIMB13064 (lane 5), and Y2 (lane 6). *Cla*I-digested total DNA of the *dhaA*-carrying gram-negative bacterium *P. pavonaceae* 170 (14) was used as a positive control (Lane 1). Lane 7, digoxigenin-labeled DNA markers of 23.1, 9.4, 6.7, 4.4, 2.3, and 2.0 kb. Hybridization signals identical in size to the hybridization signals obtained for strains NCIMB13064 and m15-3 were also observed for strain TB2 (result not shown).

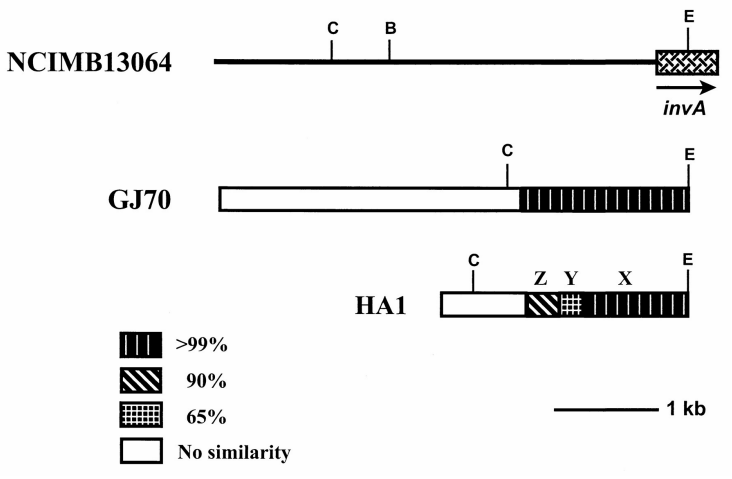

Figure 4. Schematic overview of the sequenced DNA regions upstream of the *Eco*RI site in the *invA* gene of strains NCIMB13064, GJ70, and HA1. DNA segments in strain HA1 that differ from the corresponding region in strain NCIMB13064 in about 0 to 1 (X), 10 (Z), or 35% (Y) of their positions are indicated. Open boxes, regions in strains HA1 or GJ70 that show no sequence similarity to the corresponding region in strain NCIMB13064; B, C, and E, *Bam*HI, *Cla*I, and *Eco*RI restriction sites, respectively.

The genetic organization of the *dhaA* gene regions was further examined by performing hybridization experiments with *Bam*HI- or *Eco*RI-digested DNA and an IS*2112*-specific probe. Since IS*2112* has no restriction site for *Bam*HI (Fig. 1B), a single hybridizing band corresponds to each copy of IS*2112*. Two copies of IS*2112* were present in strains NCIMB13064 and TB2, whereas one copy was present in strains GJ70, m15-3, and Y2 (results not shown). No sequences homologous to IS*2112* were found in strain HA1. We examined the regions flanking IS*2112* by digesting the genomic DNAs with *Eco*RI. Since IS*2112* has one cutting site for this enzyme (Fig. 1B), two hybridizing DNA bands were found for each copy of IS*2112* (Fig. 5). Hybridizing bands at about 6 and 2.3 kb were found in DNA from strains m15-3, NCIMB13064, and TB2. The 6-kb DNA band was identical in size to the *Eco*RI restriction fragment that includes the DNA region upstream of the *tnpA* ORF of IS*2112* in strain NCIMB13064 (see Fig. 1B). Since in strain m15-3 only one copy of IS*2112* is present, the hybridizing band at about 2.3 kb corresponds to the DNA region downstream of this copy of IS*2112*. These results indicate that the regions flanking the IS*2112* sequence that is present approximately 6 kb upstream of the *dhaA* gene in strain NCIMB13064 (Fig. 1B) are conserved in strains m15-3 and TB2. The second copy of IS*2112* in strain NCIMB13064 does not contain an *Eco*RI restriction site, indicating that this sequence is not perfectly conserved. This is in agreement with the results of Kulakov and coworkers (10), who demonstrated that the second copy of IS*2112* in strain NCIMB13064 is an iso-IS*2112* sequence containing 23 nucleotide substitutions. In total DNA of strain Y2 the 2.3-kb hybridizing DNA band was present, whereas the hybridization pattern for strain GJ70 was completely different (Fig. 5).

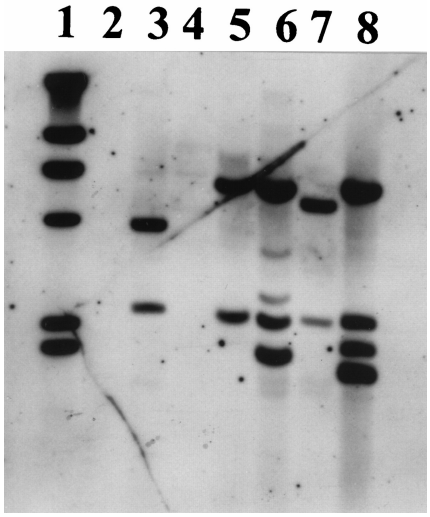

Figure 5. Results of a Southern hybridization experiment with an IS*2112*-specific probe and *Eco*RI-digested total DNA of strains GJ70 (lane 3), HA1 (lane 4), m15-3 (lane 5), NCIMB13064 (lane 6), Y2 (lane 7), and TB2 (lane 8). Lane 1 contained the same markers as lane 7 in Fig. 3. *Eco*RI-digested total DNA of *P. pavonaceae* 170 was used as a negative control (lane 2).

The hybridization and sequence data were used to construct physical maps of the *dhaA* gene regions in the six chloroalkane degraders. In summary, three classes that differ from each other with respect to the extent of the conserved *dhaA* gene segment could be defined (Fig. 6). The minimal structure present in all strains consists of a conserved region of 12.5 kb, including the regulatory gene *dhaR* and the haloalkane-degradative genes *dhaA*, *adhA*, and *aldA*, as well as the putative invertase gene *invA*. The presence of this highly conserved haloalkane catabolic gene cluster in all chloroalkane-degrading strains strongly suggests that these strains may have obtained this gene cluster as a preassembled unit from a common ancestral bacterial strain. It is noteworthy that the sequence similarity between strains NCIMB13064, m15-3, and TB2, isolated from contaminated sites in the United Kingdom, Japan, and the United States, respectively, extends to at least 22 kb (Fig. 6).

Genomic localization of the *dhaA* gene region

Since the haloalkane catabolic gene cluster of strain NCIMB13064 is located on a self-transmissible plasmid (pRTL1) (11, 15a), it is reasonable to propose that plasmid transfer has played a role in the dissemination of this gene cluster among the *Rhodococcus* strains. Using a direct lysis method, which prevents shearing of plasmid DNA, plasmids were detected in all analyzed strains (Fig. 7A). Plasmids of the same size, slightly smaller than 80-kb plasmid pRTL2 (11) of strains NCIMB13064 and S92 (a derivative of strain NCIMB13064) (11), were detected in strains Y2, TB2, m15-3, GJ70, and HA1. In strains TB2 and HA1, plasmids comparable in size to the 100-kb plasmid pRTL1 of strains NCIMB13064 and S92 were also present. In strain GJ70, additional smaller plasmids were detected (Fig. 7A).

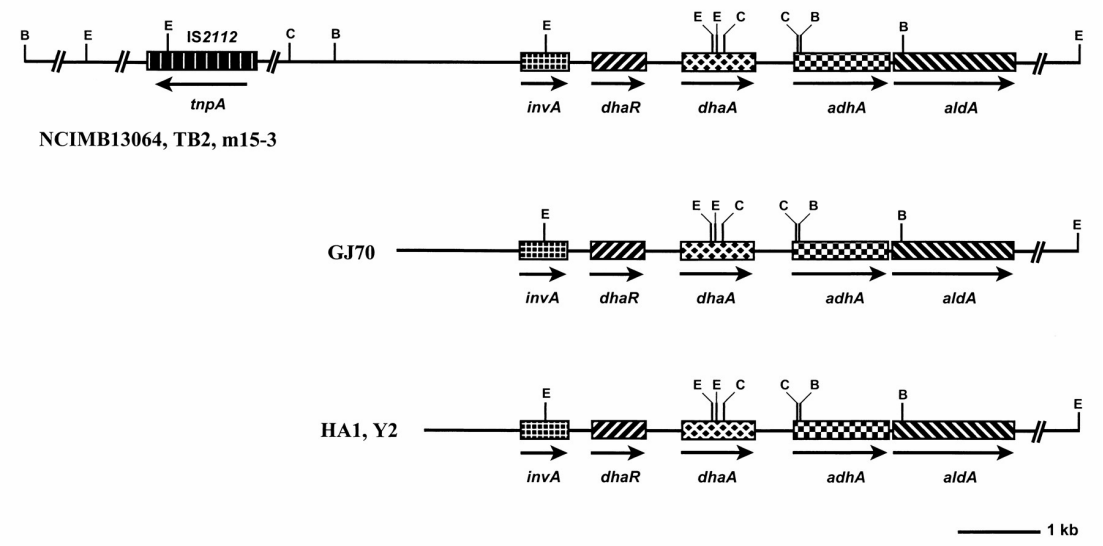

Figure 6. Physical maps of the dehalogenase gene regions of the six *Rhodococcus* strains isolated from widely separated geographical locations. Arrows indicate the direction of transcription. B, C, and E, *Bam*HI, *Cla*I, and *Eco*RI restriction sites, respectively.

In order to determine the location of the haloalkane catabolic gene cluster in the six *Rhodococcus* strains, chromosomal and plasmid DNA was hybridized with a *dhaA*-specific probe. In all strains (except S92), hybridization to both plasmid and chromosomal DNA was observed (Fig. 7B). The *dhaA* probe hybridized with plasmids of the same size (approximately 70 kb) in strains Y2, TB2, m15-3, and GJ70. For strain HA1, hybridization was detected with a plasmid whose size was the same as that of pRTL1 (compare lanes 5 to 7 in Fig. 7B). The hybridization signals observed above the plasmid bands in strains Y2, TB2, m15-3, and GJ70 are probably due to hybridization with the different forms of the same plasmid.

The occurrence of the highly conserved haloalkane catabolic gene cluster on plasmids ranging in size from 70 to 100 kb might suggest that a single mobile plasmid may have been transferred between different hosts and subsequently has undergone insertions or deletions (or vice versa). A similar phenomenon was also observed by Herrick and coworkers (5), who found that a naphthalene dioxygenase gene (*nahAc*) was present on different-size plasmids of naphthalene-degrading bacteria isolated from a coal tar-contaminated field site. Restriction fragment length polymorphism patterns and hybridization analysis indicated that all these plasmids were closely related to each other and to the naphthalene catabolic plasmid (pDTG1) of *Pseudomonas putida* NCIB 9816-4, indicating that a pDTG1-like plasmid is the mobile genetic element responsible for transferring naphthalene catabolic genes among bacteria in situ (22). Further investigations are necessary to determine whether the haloalkane catabolic gene cluster is located on pRTL1-like plasmids in all *Rhodococcus* strains analyzed here or on other plasmid backbones.

Kulakova et al. (11) demonstrated two important characteristics of plasmid pRTL1 that account for the mobility of the *dhaA* catabolic unit within the genome of strain NCIMB13064:

first, the ability of the entire pRTL1 plasmid to integrate into the bacterial chromosome, and second, the spontaneous deletion of a 20-kb fragment of pRTL1 and the subsequent integration of this fragment into the chromosome. Both events lead to the loss of haloalkane dehalogenase activity by the host cells, and in both cases the corresponding derivative strains can revert to the original pheno- and genotypes. Insertion sequence IS*2112* was shown to be involved in these genome rearrangements, although no direct link between the rearrangements and IS*2112* transposition could be demonstrated (10). Based on these observations, it is likely that plasmid integration and/or transposition events may also be responsible for positioning the haloalkane catabolic gene cluster on the chromosomes of the other *Rhodococcus* strains analyzed in this study. Integration and excision of an entire catabolic plasmid or catabolic gene unit could play an important role in gene persistence and (enhanced) expression of catabolic genes in environments where substrate resources fluctuate (9, 11).

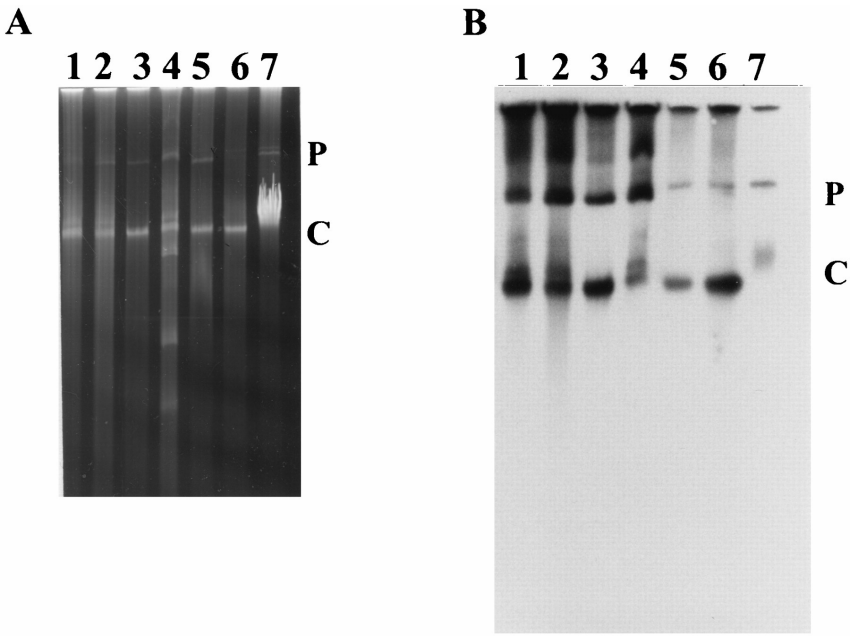

Figure 7. Visualization of plasmids of haloalkane-degrading *Rhodococcus* strains. Agarose gel (A) and autoradiogram (B) of the same gel after Southern hybridization with the *dhaA* gene as the probe. Lane 1, strain Y2; lane 2, strain TB2; lane 3, strain m15-3; lane 4, strain GJ70; lane 5, strain HA1; lane 6, strain NCIMB13064; lane 7, strain S92 (derivative of strain NCIMB13064) (11). C and P, chromosomal and plasmid DNA, respectively.

Since all gram-positive haloalkane degraders analyzed in this study were identified as rhodococci, it seems that there is a reservoir for haloalkane catabolic genes in members of this genus. However, it should be noted that these haloalkane-degrading strains were isolated by enrichment from (undiluted) environmental samples using high haloalkane concentrations. If *Rhodococcus* strains are preferentially isolated under these conditions because of their rapid

growth, this could explain why most of the haloalkane-degrading bacteria isolated from natural soil samples exhibit surprising uniformity in their phenotypes and cell structures (G. J. Poelarends, J. E. T. van Hylckama Vlieg, T. Bosma, and D. B. Janssen, unpublished data; this study). We thus may have analyzed only a small branch of cultivable gram-positive bacteria capable of degrading (synthetic) haloalkanes. In recent work, however, we reduced the possible bias during batch enrichment by directly plating bacteria from diluted environmental samples on selective plates (Poelarends et al., unpublished data). Haloalkane-degrading bacteria could be easily isolated, and most of them had the typical *Rhodococcus* morphology and contained the *dhaA* gene. Since *Rhodococcus* strains were easily isolated from the natural environment, they are clearly of ecological importance.

In summary, we have shown that the haloalkane dehalogenase gene regions of the analyzed gram-positive haloalkane-utilizing bacteria are composed of a single catabolic gene cluster distributed worldwide. The high degree of sequence similarity, the conservation of gene order, and the absence of inserted or deleted ORFs within this gene cluster in all strains suggest that the distribution process occurred recently, possibly as the result of the widespread use of synthetic haloalkanes in industry and agriculture. The data also suggest that transfer of the haloalkane catabolic gene cluster has occurred over large geographical distances, which is difficult to interpret without invoking long-distance distribution mechanisms for microorganisms. This group of bacteria should prove to be an interesting subject for studying microbial evolution and gene transfer.

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Chapter 6

The *trans*-3-chloroacrylic acid dehalogenase from *Pseudomonas pavonaceae* 170 shares structural and mechanistic similarities with 4-oxalocrotonate tautomerase

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The genes (*caaD1* and *caaD2*) encoding the *trans*-3-chloroacrylic acid dehalogenase (CaaD) of the 1,3-dichloropropene-utilizing bacterium *Pseudomonas pavonaceae* 170 were cloned and heterologously expressed in *Escherichia coli* and *Pseudomonas* sp. strain GJ1. CaaD is a hexameric protein of 50 kDa that consists of three α-subunits of 75 amino acid residues and three β-subunits of 70 residues. It catalyzes the hydrolytic cleavage of the β-vinylic carbon-chlorine bond in *trans*-3-chloroacrylic acid with a turnover number of 6.4 s^{-1}. On the basis of sequence similarity, oligomeric structure, and subunit size, CaaD appears to be related to 4-oxalocrotonate tautomerase (4-OT). This tautomerase consists of six identical subunits of 62 amino acid residues and catalyzes the isomerization of 2-oxo-4-hexene-1,6-dioate, via hydroxymuconate, to yield 2-oxo-3-hexene-1,6-dioate. In view of the oligomeric architecture of 4-OT, a trimer of homodimers, CaaD is postulated to function as a trimer of αβ-dimers. The sequence conservation between CaaD and 4-OT and site-directed mutagenesis experiments suggested that Pro-1 of the β-subunit and Arg-11 of the α-subunit are active site residues in CaaD. Pro-1 could act as proton acceptor/donor and Arg-11 is probably involved in carboxylate binding. Based on these findings, a novel dehalogenation mechanism is proposed for the CaaD-catalyzed reaction which does not involve the formation of a covalent enzyme-substrate intermediate.

INTRODUCTION

Isomer-specific 3-chloroacrylic acid dehalogenases catalyze the hydrolytic cleavage of the β-vinylic carbon-chlorine bond in either *cis*- or *trans*-3-chloroacrylic acid to yield malonic acid semialdehyde and HCl. These enzymes are produced by both gram-positive and gram-negative bacteria, including *Pseudomonas pavonaceae* 170 (29), *Pseudomonas cepacia* CAA1 (12), and the coryneform bacterial strains FG41 (49) and CAA2 (12), enabling these organisms to use one or both isomers of the xenobiotic compound 3-chloroacrylic acid for growth. The dehalogenases from strain FG41 were purified to homogeneity and the *trans*-3-chloroacrylic acid dehalogenase was found to be a 50 kDa enzyme composed of different subunits of 8.7 and 7.4 kDa, whereas the *cis*-3-chloroacrylic acid dehalogenase was an enzyme composed of two or three identical 16 kDa subunits (49). Although large fragments of these dehalogenating enzymes were sequenced, no significant sequence similarities with other protein sequences were found when the different databases were searched in 1992 (49).

Whereas most hydrolytic dehalogenases that are active with halogenated aliphatic compounds (so-called halidohydrolases), such as haloalkane dehalogenases (28, 32, 52), haloacetate dehalogenases (16-18) and 2-haloacid dehalogenases (23, 27, 33), are only able to displace halogens bound to sp^3-hybridized carbon atoms, 3-chloroacrylic acid dehalogenases are unique in that they can cleave the much more stable vinylic carbon-halogen bond, in which the halogen is bound to an sp^2-hybridized carbon atom. Cleavage of the latter can also occur with 4-chlorobenzoyl-CoA dehalogenases, but in that case activation of the substrate (4-chlorobenzoate) to its coenzyme A derivative is needed (2, 6, 54). 3-Chloroacrylic acid dehalogenases are to our knowledge the only enzymes known to dehalogenate substrates with unactivated vinylic halogens.

Nothing is known about the catalytic mechanism of 3-chloroacrylic acid dehalogenases. To obtain insight in the structure, mechanism, and ancestry of these enzymes, we sequenced the genes encoding the *trans*-3-chloroacrylic acid dehalogenase of *P. pavonaceae* 170 and characterized the expressed protein. The results indicate that the dehalogenase shares both structural and mechanistic similarities with 4-oxalocrotonate tautomerase, an enzyme involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle.

MATERIALS AND METHODS

Materials

All reagents, buffers, and solvents were obtained from Acros Organics, Merck, or

Sigma unless noted otherwise. Tryptone and yeast extract were obtained from Difco. Halogenated compounds were obtained from Acros Organics, Lancaster, Maybridge, or Aldrich, and were at least 97% pure according to the manufacturer. Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase, multipurpose agarose, the high pure plasmid isolation kit, and chemicals used for PCR amplification were purchased from Boehringer Mannheim. The QIAquick PCR purification kit and the QIAEX II gel extraction kit were obtained from Qiagen. DEAE-cellulose was obtained from Whatman Ltd. (Kent, England) and hydroxylapatite was obtained from Bio-Rad laboratories (Richmond, Calif.). A prepacked Superdex 75 gel filtration column and a low molecular weight gel filtration calibration kit were purchased from Pharmacia. Oligonucleotides were supplied by Eurosequence BV (Groningen, The Netherlands).

Bacterial strains, plasmids and growth conditions

The characteristics of *P. pavonaceae* 170, formerly known as *Pseudomonas cichorii* 170, are given elsewhere (29, 50). *Escherichia coli* JM101 (55) and plasmid pBluescript SK$^-$ (Stratagene) were used for subcloning experiments. *E. coli* HB101 (pRK600) (9) was the helper strain used for mobilizing pLAFR3-derived cosmids and pDSK519-derived plasmids in triparental matings with *Pseudomonas* sp. strain GJ1 (14). Cosmid pLAFR3 and plasmid pDSK519 are mobilizable broad-host-range vectors (19, 37). *E. coli* BL21(DE3) was used in combination with the T7 expression system (pET5a system; Promega) for overexpression of the dehalogenase and the mutant enzymes (42).

Cells for general cloning and expression were cultivated at 30°C in Luria-Bertani (LB) medium (35). When required, Difco agar (15 g/liter) was added to the medium. Antibiotics were added in the following amounts: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml.

General methods

Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods descibed by Sambrook et al. (35). Triparental matings were carried out as described elsewhere (15). DNA sequencing was performed at the BioMedical Technology Centre (Groningen, The Netherlands) using a Pharmacia ALF-Express automatic sequencing machine according to the instructions provided with the Amersham Thermo Sequenase cycle-sequencing kit. The base sequence was determined by analyzing fluorescent dye-labeled nucleotide fragments. Nucleotide sequence data were analyzed by using the programs supplied in the DNASTAR software package (DNASTAR inc., Madison WI, USA). Searches for nucleotide and amino acid sequence similarities were done by using the BLAST program (1) and the DDBJ/EMBL/Genbank databases. Amino acid sequences were aligned by using CLUSTALW (48). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under

denaturing conditions on gels containing 15 - 20% polyacrylamide. The gels were stained with Coomassie brilliant blue. Protein concentrations were estimated with Coomassie brilliant blue by using bovine serum albumin as the standard. N-terminal amino acid sequencing was performed by Eurosequence BV using chemicals, reagents, and a sequenator (Model 477A) of Applied Biosystems (Warrington, UK). The native molecular mass of the purified dehalogenase was determined by Superdex 75 gel filtration using a FPLC system according to the instructions provided with the Superdex 75 column and the low molecular weight calibration kit of Pharmacia. Circular dichroism (CD) spectra were recorded on a AVIV CD spectrometer (62A DS).

Enzyme activities

Dehalogenase assays were performed by incubating an appropriate amount of enzyme or cell extract with 3 ml of 5 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (20). All dehalogenase activities are expressed as units per milligram; 1 U was defined as the amount of enzyme that catalyzes the production of 1 μmol of halide per min. Enzyme assays were carried out twice, and the differences in specific activities were less than 10%.

Single colonies on agar plates were screened for dehalogenase activity by monitoring halide production upon incubation with *cis*- or *trans*-3-chloroacrylic acid as described previously (30).

Polymerase chain reaction

PCR reactions were carried out in a Progene DNA thermal cycler (New Brunswick Scientific Benelux B.V.). The amplification reaction mixtures (100 μl) contained standard *Taq* amplification buffer, 250 μM of each deoxyribonucleotide triphosphate, 100 ng of each primer, 100 ng template DNA, and 2 U of *Taq* DNA polymerase. The cycling parameters were 94ºC for 5 min followed by 30 cycles of 94ºC for 60 s, 58ºC for 60 s, and 72ºC for 90 s, with a final elongation step of 72ºC for 10 min. The reaction mixtures were subjected to electrophoresis in 1% agarose gels, and PCR products were stained with ethidium bromide.

Construction of expression vectors

The dehalogenase expression vector pET44T2 was made using the overlap extension polymerase chain reaction as described by Ho *et al*. (13). The external PCR primers were oligonucleotides 5'-CACGG**CATATG**CCGATGATCTCTTGCGAC-3' (primer A) and 5'-TTGCCCAAGCAGAG**GGATCC**CCTAGCT-3' (primer D). Primer A contains a *Nde*I restriction site (in bold) and anneals to the 5' end of the *caaD1* gene. Primer D contains a *Bam*HI restriction site (in bold) and anneals to the complementary sequence directly downstream of the *caaD2* gene. The internal PCR primers were oligonucleotides 5'-CATGTTATCTCCTTCATTACTTGAGTT-3' (primer B) and 5'- TCAAGTAATGAAGG-

AGATAACATGCCCTTC-3' (primer C). Primer C contains the desired mutations (underlined) that result in a new ribosome binding site (identical to the one provided by plasmid pET5a) in front of the *caaD2* gene. Primer B is the complementary primer. In two separate PCR's, the AB and CD fragments were generated using cosmid pPS41, which harbors the dehalogenase genes, as the template with primers A and B in one reaction and primers C and D in a second reaction. The PCR reaction mixtures were subjected to electrophoresis in an 1% agarose gel, and the two PCR fragments were extracted seperately using the QIAEX II gel extraction kit. Subsequently, a second PCR was carried out on a mixture of the AB and CD fragments using primers A and D. The mutated DNA fragment was isolated from an 1% agarose gel. The restriction sites *Nde*I and *Bam*HI were used to clone this DNA fragment into plasmid pET5a for overexpression of the dehalogenase under control of the T7 promoter. The newly constructed plasmid, pET44T2, was sequenced in order to verify the mutations in front of the *caaD2* gene.

A dehalogenase expression vector for *Pseudomonas* sp. strain GJ1 was constructed by cloning the *Sal*I fragment of cosmid pPS41 into the *Sal*I linearized broad-host-range vector pDSK519, resulting in pDSKcaaD. Upon introduction of this vector into *Pseudomonas* sp. strain GJ1, high level expression of the dehalogenase gene under control of its own promoter was obtained.

Site-directed mutagenesis

The *trans*-3-chloroacrylic acid dehalogenase mutants were constructed using the coding sequence for the dehalogenase in plasmid pET44T2 as the template. The αP1A, αR11A, and αR11K mutants were generated by PCR using the primers 5'-CACGG**CATATG***GCG*ATGATCTCTTGCGAC-3'; 5'-CACGG**CATATG**CCGATGATC-TCTTGCGACATGCGCTATGGG*GCC*ACAGACGAACAA-3'; and 5'-CACGGC**CATAT-G**CCGATGATCTCTTGCGACATGCGCTATGGG*AAA*ACAGACGAACAA-3';
respectively. These primers anneal to the 5' end of the wild-type coding sequence and were used in combination with primer D. They all contain a *Nde*I restriction site (in bold) and the codon for the desired mutation (in italics). The αF39A, αF39Y, and βP1A mutants were generated by overlap extension PCR. Primers A and D were used as the external PCR primers. For the αF39A mutant, the internal PCR primers were oligonucleotides 5'-GAGCCCCGCGAGAACATT*GCC*TTTGTGATT-3' (mutated codon in italics) and 5'-AATGTTCTCGCGGGGCTC-3' (primer E). For the αF39Y mutant, the internal PCR primers were oligonucleotide 5'-GAGCCCCGCGAGAACATT*TAC*TTTGTGATT-3' (mutated codon in italics) and primer E. For the βP1A mutant, the internal PCR primers were oligonucleotides 5'-TCAAGTAATGAAGGAGATAACATG*GCC*TTC-3' (mutated codon in italics) and 5'-CATGTTATCTCCTTCATTACTTGAGTT-3'.

PCR reactions were carried out as described above and PCR products were purified

using the QIAquick PCR purification kit or the QIAEX II gel extraction kit. The restriction sites *Nde*I and *Bam*HI that were introduced during the amplification reactions were used to clone the PCR products into plasmid pET5a for overexpression of the dehalogenase mutants. The cloned dehalogenase genes were sequenced in order to verify the mutations.

Preparation of crude extracts

The *trans*-3-chloroacrylic acid dehalogenase and the mutant enzymes were expressed in *E. coli* BL21(DE3) using the pET system. Fresh BL21(DE3) transformants containing the desired plasmid were collected from a plate by resuspending them in 1 ml of LB medium and were used to inoculate 100 ml of LB/ampicillin medium to a starting OD_{600} of 0.1. After overnight growth at 30ºC, cells were harvested by centrifugation (10 min at 10,000 × *g*), washed with 1 volume of 50 mM Tris-sulfate buffer (pH 8.2), and disrupted at 4ºC in an appropriate amount of this buffer by sonication (10 s per ml of suspension at a 70 W output in a Vibra cell sonicator). A crude extract was obtained by centrifugation (45 min at 16,000 × *g*).

Purification of the dehalogenase

For isolation of the *trans*-3-chloroacrylic acid dehalogenase of *P. pavonaceae* 170, a single colony of strain 170 was used to inoculate 100 ml of LB medium. After overnight growth at 30°C, the culture was used to inoculate 1 liter of LB medium. This culture was grown at 30°C until the early stationary growth phase. Cells were harvested by centrifugation (10 min at 10,000 × *g*), washed with 1 volume of TEMAG buffer (10 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, 10% glycerol, pH 8.0), and stored at -20°C until further use. Preparation of a crude extract and purification of the dehalogenase were done as described below for the recombinant enzyme.

High level *trans*-3-chloroacrylic acid dehalogenase expression was obtained in *Pseudomonas* sp. strain GJ1. A single colony of strain GJ1 containing the expression vector pDSKcaaD was used to inoculate 10 ml of LB/kanamycin medium. After overnight growth at 30ºC, the culture was used to inoculate 1 liter of LB/kanamycin medium. This culture was also grown overnight at 30ºC. Cells were harvested by centrifugation (10 min at 10,000 × *g*), washed with 1 volume of TEMAG buffer, and stored at -20ºC.

The *trans*-3-chloroacrylic acid dehalogenase was purified to homogeneity by using a modification of a published procedure (49). In a typical experiment, cells of a 1 liter-culture were thawed and suspended in 20 ml of TEMAG buffer. The cells were disrupted at 4 to 10°C by continous sonication, after which unbroken cells and debris were removed by centrifugation for 1 h at 50,000 rpm in a type 70 Ti rotor (Beckman). The supernatant was applied to a DEAE-cellulose column which had previously been equilibrated with TEMAG buffer. The column was washed with 1 column volume of TEMAG buffer, and the proteins were eluted with a lineair gradient of 0 to 0.5 M ammonium sulfate in TEMAG. Fractions that

showed the highest dehalogenase activity with *trans*-3-chloroacrylic acid were pooled and dialyzed overnight against PEMAG buffer (5 mM potassium phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, 10% glycerol, pH 6.5). The dialysate was loaded onto a hydroxylapatite column which had previously been equilibrated with PEMAG buffer. The column was washed with 1 column volume of PEMAG buffer, and the proteins were eluted with a linear gradient of 5 to 100 mM potassium phosphate in PEMAG. Fractions with the highest *trans*-3-chloroacrylic acid dehalogenase activity were analyzed by SDS-PAGE, and those that contained purified enzyme were pooled and dialyzed against TEMAG buffer. The enzyme was stored at 4°C or -20°C.

Nucleotide sequence accession number

The nucleotide sequence of the dehalogenase gene region has been deposited in the Genbank database under accession number AJ290446.

RESULTS

Cloning and characterization of the genes encoding the *trans*-3-chloroacrylic acid dehalogenase

The *trans*-specific 3-chloroacrylic acid dehalogenase of *P. pavonaceae* 170 was partially (~75%) purified. After SDS-PAGE (data not shown), two dominant protein bands at about 7.5 and 8.5 kDa were observed, indicating that the dehalogenase consisted of two different subunits. Both subunits were subjected to N-terminal sequence analysis. The sequence of the 8.5 kDa subunit (designated α-subunit) was established as P-M-I-S-C-D-M-R-Y-G-R-T-D-E-Q-K and that of the 7.5 kDa subunit (designated β-subunit) as P-F-I-E-C-H-I-A-T-G-L-S-V-A-R-K-Q-Q-L-I-R-D.

To isolate the genes encoding the *trans*-3-chloroacrylic acid dehalogenase, individual clones of a previously constructed cosmid library of *P. pavonaceae* 170 (31) were screened for dehalogenase activity by monitoring halide production upon incubation with *trans*-3-chloroacrylic acid. Out of 2,500 *E. coli* HB101 clones tested, 1 clone expressing the *trans*-3-chloroacrylic acid dehalogenase was found. The recombinant cosmid (pPS41) encoding the dehalogenase was isolated and the localization of the dehalogenase genes was determined by screening subclones for dehalogenase activity.

Nucleotide sequencing of a 2.6 kb *Sal*I subclone of pPS41 revealed several open reading frames (Fig. 1). The two open reading frames encoding the *trans*-3-chloroacrylic acid dehalogenase (designated *caaD1* and *caaD2*) were identified by using the N-terminal amino acid sequences determined for both subunits of the enzyme isolated from *P. pavonaceae* 170. These N-terminal amino acid sequences are identical to those predicted from the DNA

sequence if the initiating *N*-formyl-methionines are removed during posttranslational processing. The *caaD1* gene encodes a protein (the α subunit) of 76 amino acids with a calculated molecular weight (M_r) of 8.47 kDa. The *caaD2* gene was found downstream of *caaD1* and encodes a protein (the β subunit) of 71 amino acids (M_r, 7.64 kDa). The M_r's of both subunits are in agreement with their masses estimated from SDS-PAGE.

Screening of the cosmid library of *P. pavonaceae* 170 did not reveal clones that expressed the *cis*-3-chloroacrylic acid dehalogenase. To check for the occurrence of a *cis*-3-chloroacrylic acid dehalogenase gene or other genes related to 3-chloroacrylic acid utilization in the vicinity of the *caaD1* and *caaD2* genes, the DNA regions flanking these genes were analyzed. An open reading frame, designated *orf130*, encoding a putative protein of 130 amino acids was found downstream of *caaD2* (Fig. 1). Orf130 shares considerable similarity with the hypothetical proteins Orf129 (53% identity) and YodA (51% identity) of *Bacillus subtilis* (22). However, the possible functions of these hypothetical proteins have not been reported. Orf130 also shares notable similarities with the *caaD1* gene product including an N-terminal proline and a short stretch of sequence, YGRTD(A,E)Q, which they have in common. This notion prompted us to test if the *orf130* gene codes for the *cis*-3-chloroacrylic acid dehalogenase. Crude extract of *E. coli* cells overexpressing Orf130 did not show dehalogenase activity toward *cis*-3-chloroacrylic acid. Orf130 was also not capable of dehalogenating *trans*-3-chloroacrylic acid nor of converting *cis*-3-chloroacrylic acid into *trans*-3-chloroacrylic acid. These results indicate that the *orf130* gene product is not involved in 3-chloroacrylic acid utilization. Downstream of *orf130* the beginning of an open reading frame (*orf4*) was found that codes for a peptide of 178 amino acids that shares extensive similarity with the N-termini of the putative alcohol dehydrogenases YhdH (56% identity) of *E. coli* (3) and YhfP (47% identity) of *B. subtilis* (22). In the 1 kb region upstream of the *caaD1* gene, no open reading frames encoding putative proteins with significant similarity to protein sequences present in the current databases were found.

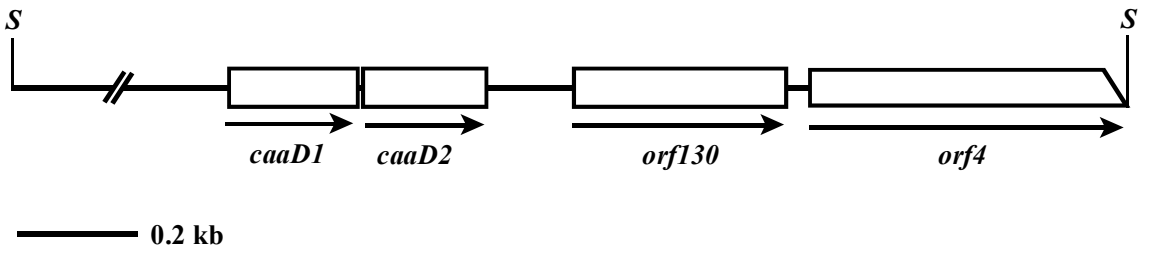

Figure 1. Organization of the *trans*-3-chloroacrylic acid dehalogenase gene region of *P. pavonaceae* 170. Genes are shown as open boxes and arrows indicate the direction of transcription. *Sal*I restriction sites (S) are shown.

Expression and characterization of the dehalogenase

E. coli HB101 harboring recombinant cosmid pPS41 displayed poor expression of the *trans*-3-chloroacrylic acid dehalogenase (Table 1). Introduction of pPS41 into *Pseudomonas* sp. strain GJ1 resulted in significantly better expression (Table 1). To obtain overexpression of the dehalogenase in strain GJ1, we cloned the *Sal*I fragment that harbors the dehalogenase genes (Fig. 1) into the *Pseudomonas* high-copy number vector pDSK519, resulting in pDSKcaaD. The coding sequence for the dehalogenase in pDSKcaaD is under control of its own promoter and the dehalogenase was expressed constitutively in strain GJ1 in a soluble and active form up to a concentration equivalent to 13 % of the total soluble cellular protein (Table 1). The dehalogenase was isolated from strain GJ1(pDSKcaaD) with a yield of 20 mg of pure protein, as judged by SDS-PAGE, from a 1-liter culture. The purified enzyme could be stored for several months in TEMAG buffer at 4°C or -20°C without significant loss of activity.

Table 1. Expression of the *trans*-3-chloroacrylic acid dehalogenase in *E. coli* and *Pseudomonas*

Organism	Dehalogenase sp act[a] (mU/mg of protein)
E. coli strains	
HB101 (pPS41)	30
BL21(DE3) (pET44T2)	1500
Pseudomonas strains	
P. pavonaceae 170	310
GJ1 (pPS41)	400
GJ1 (pDSKcaaD)	2380[b]

[a]Specific activities for *trans*-3-chloroacrylic acid (5 mM) were determined with crude extracts prepared from cells grown in Luria Broth.

[b]The specific activity measured in crude extract from strain GJ1(pDSKcaaD) corresponds to a dehalogenase concentration equivalent to 13% of the total soluble cellular protein, based on a specific activity of 18,000 mU/mg for the purified enzyme.

The native molecular mass of the dehalogenase was estimated by gel filtration chromatography to be 50 kDa. A comparison of this value to the subunit molecular masses (8.47 kDa and 7.64 kDa) determined from the primary amino acid sequences suggests that the native dehalogenase is a hexameric protein, probably consisting of three α- and three β-

subunits ($\alpha_3\beta_3$).

The dehalogenase catalyzed the liberation of halide from *trans*-3-chloroacrylic acid (specific activity of 18 U/mg of protein) and *trans*-3-bromoacrylic acid (48 U/mg), but showed no detectable dehalogenase activity (<0.005 U/mg) toward *cis*-3-chloroacrylic acid, indicating that the enzyme is completely isomer-selective. The saturated analog 3-chloropropionic acid was also not dehalogenated, indicating that the halogen atom is only removed when attached to an unsaturated carbon atom. Since no activity was found for 2-bromoacrylic acid and 2-chloroacrylic acid, it is essential that the halogen substituent is located at the β-position. The enzyme was also not active with *trans*-3-chloroallyl alcohol and *trans*-1,3-dichloropropene, indicating the importance of the presence of a carboxyl group in the substrate.

The dehalogenase showed a broad pH optimum around 8.5 and the temperature optimum was 40°C. By measuring the initial velocities of product formation at different *trans*-3-chloroacrylic acid concentrations, a K_m of 0.19 mM and k_{cat} of 6.4 s^{-1} were found.

Sequence similarity with 4-oxalocrotonate tautomerases/isomerases

Database searches identified seven related proteins as sharing significant sequence similarity with the *trans*-3-chloroacrylic acid dehalogenase (CaaD) (Table 2). Two are well-studied enzymes involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle, the 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida* mt-2 (41) and the 73% identical isozyme from *Pseudomonas* sp. CF600 (43). Both are hexameric proteins that consist of identical subunits of 62 amino acid residues (5) and catalyze the isomerization of 2-oxo-4-hexene-1,6-dioate, via hydroxymuconate, to yield 2-oxo-3-hexene-1,6-dioate (53). The other five proteins that were retrieved from the similarity search have not been studied, but based on sequence similarity to 4-OT they might be classified as putative 4-oxalocrotonate tautomerases/isomerases. Pairwise identities among the seven identified 4-OT homologues range from 35 to 92%.

Alignments optimizing identity between the amino acid sequences of the two dehalogenase subunits and the seven 4-OT homologues are shown in Fig. 2. Pairwise identities between the α-subunit (CaaD1) of the dehalogenase and the 4-OT sequences fall between 23 and 35% (Table 2), with the highest sequence similarity in the N-terminal region, particularly in the region boxed in Fig. 2A. Pairwise identities between the β-subunit (CaaD2) and the 4-OT sequences are lower, and range from 16 to 25% (Table 2).

The sequence alignment of Fig. 2 suggests catalytic residues for CaaD that are in the same position in the alignment as the identified catalytic residues of 4-OT. Affinity labeling (38), kinetic analysis (39), chemical synthesis (10), NMR (40, 41), site-directed mutagenesis (7), and crystallographic studies (43) identified the amino-terminal proline as the catalytic base in the 4-OT-catalyzed tautomerization reaction. This N-terminal proline is invariant

among all identified 4-OT homologues (Fig. 2). Both dehalogenase subunits also possess an N-terminal proline, indicating that one of these prolines may serve as the catalytic base in the CaaD-catalyzed dehalogenation reaction. The second catalytic residue of 4-OT, Arg-11, which is absolutely conserved among the 4-OT homologues (Fig. 2), was proposed to interact with the 6-carboxylate of the substrate (2-oxo-4-hexene-1,6-dioate) to facilitate both substrate binding and catalysis (11, 43). The sequence similarity indicates that Arg-11 in the α-subunit of the dehalogenase may perform an analogous role by interacting with the carboxylate group of *trans*-3-chloroacrylic acid. The third catalytic residue of 4-OT, Arg-39, which was proposed to interact with the 1-carboxylate and the 2-keto group of the substrate to promote carbonyl polarization and catalysis (11, 43), is not conserved in the dehalogenase sequence. This seems plausible since the dehalogenase substrate contains only one carboxylate group.

Table 2. Protein sequences that are similar to *trans*-3-chloroacrylic acid dehalogenase

Enzyme	Gene	Length (aa)	Organism	Identity to α-subunit[a]	Identity to β-subunit[a]	Accession number
4-OT	*xylH*	62	*P. putida* mt-2	26%	25%	Q01468
4-OT	*dmpI*	62	*Pseudomonas* sp. CF600	26%	22%	P49172
4-OT	*ywhB*	61	*B. subtilis* 168	35%	16%	CAB02512
4-OT	*nahJ*	62	*P. stutzeri* AN10	26%	24%	AAD02155
4-OT	*xylH*	79	*S. aromaticivorans* F199	25%	22%	AAD03991
4-OT	*phnL*	76	*Pseudomonas* sp. DJ77	23%	24%	AAD03836
4-OT	*nahJ*	62	*P. putida* G7	26%	22%	AAD13221

[a]Pairwise identities between the α- or β-subunit of the dehalogenase and the 4-OT sequences were calculated by using CLUSTALW.

Characterization of dehalogenase mutants

In the homohexameric ($\alpha 6$) 4-OT molecule, Pro-1 is important for tautomerase activity since it serves as the catalytic base. To test if both Pro-1 in the α-subunit and Pro-1 in the β-subunit of the heterohexameric ($\alpha_3\beta_3$) CaaD molecule are important for dehalogenase activity, each proline was replaced by an alanine. The wild type and mutant enzymes were expressed in *E. coli* BL21(DE3) and their specific activities with *trans*-3-chloroacrylic acid and *trans*-3-bromoacrylic acid were measured in crude extracts. Mutation of Pro-1 to alanine in the β-subunit essentially abolished catalytic activity of the dehalogenase, whereas mutation of Pro-1 to alanine in the α-subunit had no significant influence on activity (Table 3). To

determine whether the loss of catalytic activity in the βP1A mutant is due to the specific alteration of the catalytic residue or to the loss of native-like protein structure, this mutant was purified to homogeneity and analyzed by gel filtration chromatography and circular dichroism (CD). The purified βP1A mutant showed an activity of 10 mU/mg of protein with *trans*-3-chloroacrylic acid, which is 1800-fold lower than the activity of wild-type enzyme. The CD spectrum of this mutant was nearly identical to that recorded for wild type, indicating that the mutation did not result in any gross conformational change (data not shown). It was further shown by gel filtration chromatography that the native molecular mass for mutant βP1A is comparable to that of wild type, indicating that the hexameric association was still intact. Hence, the 1800-fold decrease in activity found with the βP1A mutant is probably due to a direct effect of the substitution of the catalytically important proline for alanine. Taken together, the results indicate that of the two N-terminal prolines in the dehalogenase, only the N-terminal proline in the β-subunit is important for the dehalogenase-catalyzed reaction.

A

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                    +         +                    +
CaaD1 (Ppa, 170)    PMISCDMRYGRTDEQKRALSAGLLRVISEATGEPRENIFFVIREGSGINFVEHGEHLPDYVPGNANDKALIAKLK---- 75
4-OT  (Bs, 168)     PYVTVKMLEGRTDEQKRNLVEKVTEAVKETTGASEEKIVVFIEEMRKDHYAVAGKRLSDME------------------ 61
4-OT  (Psp, CF600)  PIAQLYIIEGRTDEQKETLIRQVSEAMANSLDAPLERVRVLITEMPKNHFGIGGEPASKVR--------R--------- 62
4-OT  (Pp, mt-2)    PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKVR--------R--------- 62
4-OT  (Ps, AN10)    PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMPKVHFGIGGESAKAIG--------R--------- 62
4-OT  (Pp, G7)      PIAQLYILEGRSDEQKETLIREVSEAMSRSLDAPIERVRVIITEMPKNHFGIGGEPASKLN--------R--------- 62
4-OT  (Sa, F199)    PIIEVNLFEGRPPEAKERLIKALTDAAIDAIGAPRESVRVILREMAPAHFAVGGLSFAAKAAAAAAAQPRVEEDWSNEN 79
4-OT  (Psp, DJ77)  PIIEVNLLEGRPPEAKERLIRALTDAAIGAIGAPRESVRVILREMDPAHFAVGGVSFAAK---AAAAQPRVEEDWSNEA 76
                    *         **  * *  *                          *         *
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B

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                    +         +                    +
CaaD2 (Ppa, 170)    PFIECHIATGLSVARKQQLIRDVIDVTNKSIGSDPKIINVLLVEHAEANMSISGRIHGEAASTERTPAVS--------- 70
4-OT  (Bs, 168)     PYVTVKMLEGRTDEQKRNLVEKVTEAVKETTGASEEKIVVFIEEMRKDHYAVAGKRLSDME------------------ 61
4-OT  (Psp, CF600)  PIAQLYIIEGRTDEQKETLIRQVSEAMANSLDAPLERVRVLITEMPKNHFGIGGEPASKVRR----------------- 62
4-OT  (Pp, mt-2)    PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKVRR----------------- 62
4-OT  (Ps, AN10)    PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMPKVHFGIGGESAKAIGR----------------- 62
4-OT  (Pp, G7)      PIAQLYILEGRSDEQKETLIREVSEAMSRSLDAPIERVRVIITEMPKNHFGIGGEPASKLNR----------------- 62
4-OT  (Sa, F199)    PIIEVNLFEGRPPEAKERLIKALTDAAIDAIGAPRESVRVILREMAPAHFAVGGLSFAAKAAAAAAAQPRVEEDWSNEN 79
4-OT  (Psp, DJ77)  PIIEVNLLEGRPPEAKERLIRALTDAAIGAIGAPRESVRVILREMDPAHFAVGGVSFAAK---AAAAQPRVEEDWSNEA 76
                    *         *     *  *                    *   *         *
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C

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CaaD1     (Ppa, 170)      PMISCDMRYGRTDEQKRALSAGLLRVISEATGEPRENIFFVIREGSGINFVEHGEHLPDYVPGNANDKALIAKLK 75
α-subunit (Csp, FG41)     SIIS?DMREGRTDDQKRALSG?LIEAV?NVTGEP
                            ** *** **** ******  *       ****

CaaD2     (Ppa, 170)      PFIECHIATGLSVARKQQLIRDVIDVTNKSIGSDPKIINVLLVEHAEANMSISGRIHGEAASTERTPAVS 70
β-subunit (Csp, FG41)     PFIEIRLPKPL
                          ****      *
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Figure 2. Alignments of the amino acid sequences of the α-subunit (CaaD1) or β-subunit (CaaD2) of the *trans*-3-chloroacrylic acid dehalogenase (CaaD) with the seven 4-OT sequences (A, B), or with the amino-terminal sequences of the subunits of the *trans*-3-chloroacrylic acid dehalogenase isolated from strain FG41 (C). Residues conserved throughout all sequences are indicated by an asterisk. Dashes represent residues absent in other sequences. The catalytically important residues in 4-OT are marked with + and are shown in boldface. The region of highest sequence identity among CaaD1 and the 4-OT sequences is boxed.

Table 3. Activities of wild type and mutant 3-chloroacrylic acid dehalogenase for *trans*-3-chloroacrylic acid (CAA) and *trans*-3-bromoacrylic acid (BAA)

	Dehalogenase sp act[a] (mU/mg of protein)	
Enzyme	CAA	BAA
Wild type	1500	3250
αP1A	1260	3050
βP1A	<5	15
αR11A	<5	<5
αR11K	110	240
αF39A	350	480
αF39Y	240	300

[a]Activities were measured in cell free extracts containing similar amounts of dehalogenase. Halide production with 5 mM substrate was determined at 30°C and pH 8.2.

Although CaaD has a low overall identity to the know 4-OTs, the region around Arg-11 is highly conserved (Fig. 2A). In 4-OT, this region is related to binding of the carboxylate group of the substrate (see above). The dehalogenase substrate also contains a carboxylate group, probably providing the evolutionary pressure to preserve this site in CaaD. To probe the role of Arg-11 in the catalytic activity of CaaD, this residue was mutated to alanine or lysine. Mutation of Arg-11 to alanine resulted in an inactive enzyme, indicating that Arg-11 is essential for dehalogenase activity (Table 3). The mutant αR11K had partially restored catalytic activity compared to the catalytically inactive αR11A mutant, suggesting that a positive charge at this position is important for carboxylate binding.

In many reactions involving carbon-halogen bond cleavage, the carbon-halogen bond is weakened by functional groups that interact with the halogen substituent (28, 34, 51, 52). In the α-subunit of the *trans*-3-chloroacrylic acid dehalogenase, Phe-39 is in the same position in the alignment as the catalytically important Arg-39 of 4-OT, suggesting that Phe-39 may be one of the residues that promotes carbon-halogen bond cleavage by interacting with the chlorine atom of the dehalogenase substrate. To test if Phe-39 is catalytically important, this residue was mutated to alanine or tyrosine. The αF39A and αF39Y mutants were still able to catalyze halide release from both dehalogenase substrates, although 5- to 10-fold slower compared to the wild type enzyme (Table 3), indicating that Phe-39 is not essential for dehalogenase activity.

DISCUSSION

The enzyme *trans*-3-chloroacrylic acid dehalogenase (CaaD) is elaborated by the soil bacterium *P. pavonaceae* 170 as part of a degradative pathway for the xenobiotic nematocide *trans*-1,3-dichloropropene (29). This hydrolytic dehalogenase, of which the properties are reported in this work, shares no sequence similarity with other halidohydrolases but appears to be related to the family of 4-oxalocrotonate tautomerases. No other bacterial 3-chloroacrylic acid dehalogenase genes have been cloned, but the N-terminal sequences of the subunits of the *trans*-3-chloroacrylic acid dehalogenase isolated from the gram-positive coryneform bacterial strain FG41 (49) share extensive similarity with the N-terminal parts of the CaaD subunits (Fig. 2C), suggesting that these two proteins have a common evolutionary origin. As might be expected, the two *trans*-3-chloroacrylic acid dehalogenase sequences are more related to each other than to the 4-oxalocrotonate tautomerases.

The primary amino acid sequence of 4-oxalocrotonate tautomerase (4-OT) shows no apparent similarity with those of the mammalian enzymes D-dopachrome tautomerase (DDT) (44) and macrophage migration inhibitory factor (MIF) (45), nor with that of the bacterial enzyme 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (43), but, remarkably, these four proteins share a common structural architecture (26, 44). DDT, MIF and CHMI have an almost identical subunit topology with two βαβ motifs related by pseudo-2-fold symmetry and trimeric β-sheet packing (26, 43, 44). While CHMI, MIF, and DDT are functional as homotrimers, 4-OT is a hexamer of identical monomers. The 4-OT subunit is composed of only 62 residues and is dimerized by 2-fold symmetry to form a structure similar to that of the CHMI, MIF and DDT monomer. Therefore, 4-OT is a trimer of homodimers that shows 32 symmetry; its overall hexameric structure is very similar to the trimeric structure of CHMI, MIF, and DDT (43-45). An interesting difference between the four structures is that because of the higher symmetry of the 4-OT hexamer there are potentially six active sites in 4-OT, yet only three are conserved in CHMI, MIF, and DDT (43, 44).

One of the characteristics of this superfamily of 4-OT related proteins is that its members possess an amino-terminal proline that is located at the bottom of a hydrophobic pocket. CHMI and 4-OT utilize this proline as a catalytic base in their isomerization reactions (7, 10, 38-41, 43). Pro-1 of MIF is required for its D-dopachrome tautomerase and phenylpyruvate tautomerase activities (24, 36). The N-terminal proline of DDT is proposed to serve as the catalytic base in the DDT-catalyzed tautomerization reaction (44). The Pro-1 residue is conserved among all known homologues of 4-OT (Fig. 2), MIF (24, 46), and DDT (44), and is also conserved in both subunits of CaaD (Fig. 2). In the *trans*-3-chloroacrylic acid dehalogenase isolated from strain FG41, however, the amino-terminal proline is present only in the β-subunit (Fig. 2C). This suggests that Pro-1 of the β-subunit may serve as a catalytic base in both of the *trans*-3-chloroacrylic acid dehalogenating enzymes. Site-directed

mutagenesis experiments in which the amino-terminal prolines in CaaD were replaced by alanines indeed demonstrated that Pro-1 of the β-subunit is catalytically important, whereas Pro-1 of the α-subunit does not seem to play a role in catalysis.

On the basis of its sequence similarity to 4-OT, we conclude that CaaD also belongs to the superfamily of 4-OT related proteins (26). The relatedness between CaaD and 4-OT is most apparent from the presence of a short stretch of sequence, GR(T,S)DEQK, that they have in common (Fig. 2A). This sequence motif, which includes the catalytically important Arg-11 in both 4-OT and CaaD, is not conserved in MIF and DDT, but is present as GRSlEsr around the equivalent, catalytically important, Arg-71 residue in CHMI. This functional motif is related to binding of the carboxylate group of the 4-OT, CHMI, and CaaD substrates.

In view of the oligomeric architecture of 4-OT, a trimer of homodimers (43), CaaD is postulated to function as a trimer of αβ-dimers. In contrast to the presence of potentially six active sites in the highly symmetrical 4-OT molecule (47), there are three potential active sites in CaaD. From the crystal structure of 4-OT inactivated by 2-oxo-3-pentynoate (47), the substrate should interact with Pro-1 of one subunit and Arg-11 from an adjacent subunit within the same homodimer. Consistent with this, the catalytically important Pro-1 of CaaD is located on the β-subunit, whereas Arg-11 is located on the α-subunit, showing that residues from both subunits contribute to the dehalogenase active site.

In 4-OT, Arg-11 interacts with the 6-carboxylate of the substrate (2-oxo-4-hexene-1,6-dioate) to facilitate substrate binding and catalysis, and Pro-1 transfers protons from C-3 to C-5 (Fig. 3A). We propose similar roles for Pro-1 and Arg-11 in CaaD, which leads to a minimal catalytic mechanism for the CaaD-catalyzed hydrolytic dehalogenation of *trans*-3-chloroacrylic acid (Fig. 3B). In principle, one can write several possible chemical pathways for the CaaD-catalyzed reaction, including a nucleophilic substitution and an addition-elimination mechanism. Although a one-step displacement of the halide by a hydroxyl ion has been suggested for the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids by haloacid dehalogenase (DL-DEX 113) from *Pseudomonas* sp. 113 (27), it seems unlikely with the halide bound to an sp^2-hybridized carbon atom as in *trans*-3-chloroacrylic acid. Therefore, we propose that, in parallel to the hydration of monofluorofumarate by fumarase (25), CaaD catalyzes nucleophilic addition of a hydroxyl group on an sp^2-hybridized carbon atom. This nucleophilic addition is favoured over an electrophilic reaction because of the presence of the electron-withdrawing halogen and the carboxyl group. In this scenario, Pro-1 serves as the catalytic base that activates a water molecule to attack the β-carbon atom of the substrate. This leads to two hypothetical pathways, one involving the formation of the intermediate 3-chloro-3-hydroxypropanoic acid (route 1 in Fig. 3B) and the other involving the formation of a carbanion intermediate (route 2 in Fig. 3B). Product formation from either intermediate involves redirection of electrons with departure of Cl^- and protonation at C-2. The latter suggests the presence of an acidic residue, which may be the protonated proline or another

amino acid. Loss of a proton from the hydroxyl at C-3 would produce the malonic acid semialdehyde, which may be facilitated by water or another proton acceptor. Hydration of monofluorofumarate by fumarase also yielded an unstable intermediate, α-fluorohydrin (α-fluoromalate), which subsequently decomposes to oxaloacetate and HF (25).

Because CaaD catalyzes a dehalogenation reaction, it is anticipated that functional goups involved in halogen/halide binding are required in addition to Pro-1 and Arg-11. We speculate that Phe-39 in CaaD, which is in the same position in the alignment as Arg-39 in 4-OT (Fig. 2), may interact with the chlorine atom of the substrate to promote carbon-halogen bond cleavage. Indeed, aromatic ring systems are known to be partially positively charged in the plane of the ring (4). Phenylalanine residues were also proposed to contribute to halogen/halide binding in haloalkane dehalogenase (DhlA) and L-2-haloacid dehalogenase (DhlB) from *X. autotrophicus* GJ10 (8, 34). However, the F39A and F39Y mutants of CaaD still had some residual activity, indicating that this residue is not essential. The presence of other functional groups interacting with the halogen atom of the substrate could explain why these mutants retained some activity. Indeed, in DhlA and DhlB the halogen/halide binding site is formed by more than one residue (21, 34, 51).

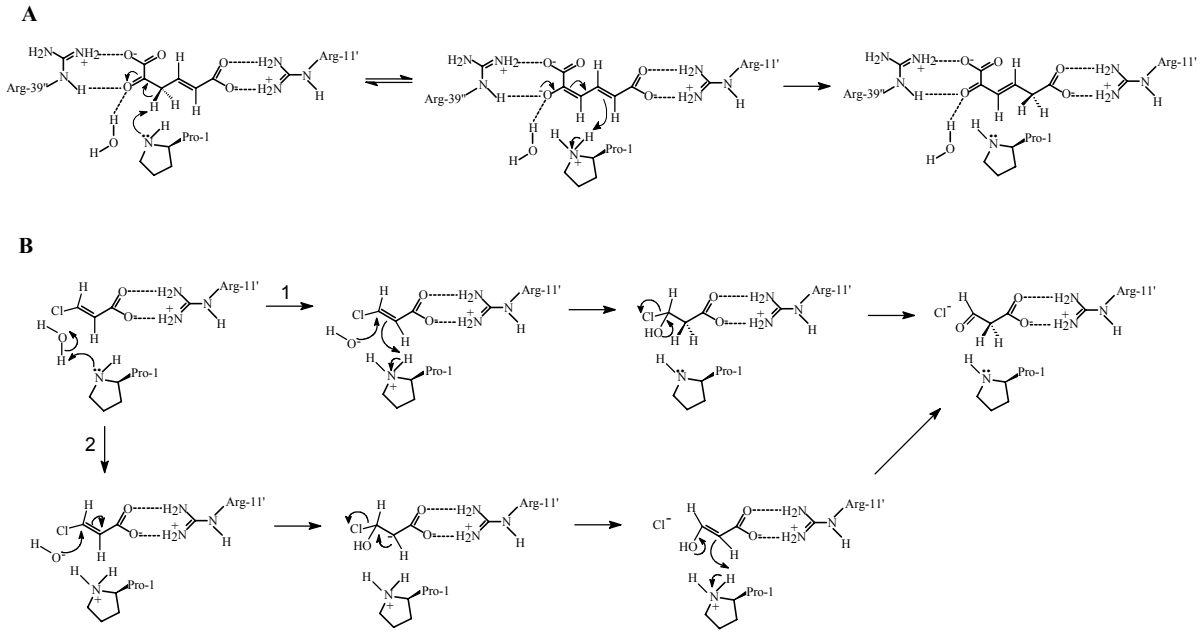

Figure 3. A comparative reaction scheme for 4-oxalocrotonate tautomerase (4-OT) and *trans*-3-chloroacrylic acid dehalogenase (CaaD). The primed residues come from other subunits. A, the reaction catalyzed by 4-OT (adopted from references 11 and 43). B, the two proposed reaction schemes for CaaD, one involving the formation of 3-chloro-3-hydroxypropanoic acid (route 1) and the other involving the formation of a carbanion intermediate (route 2). Pro-1 is shown as the catalytic base to activate a water molecule that attacks the substrate. Arg-11 is implicated in substrate binding.

Screening of the cosmid library of *P. pavonaceae* 170 did not reveal clones that expressed the *cis*-3-chloroacrylic acid dehalogenase. Thusfar, the only sequence information available for *cis*-specific 3-chloroacrylic acid dehalogenases is the N-terminal sequence of the enzyme isolated from the coryneform bacterial strain FG41 (49). This enzyme is probably a trimeric protein of 16.2 kDa subunits, and a comparison of its amino-terminal sequence with those of the *trans*-3-chloroacrylic acid dehalogenases from strains FG41 and 170 revealed no overall similarity, but showed that Pro-1 and Arg-11 are conserved (data not shown). Therefore, *cis*- and *trans*-specific 3-chloroacrylic acid dehalogenases may catalyze the dehalogenation of their respective 3-chloroacrylic acid isomers through the same mechanism as shown in Fig. 3. This mechanism is different from that of most other hydrolytic dehalogenases in that it does not involve the formation a covalent enzyme-substrate intermediate.

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Chapter 7

Summary and concluding remarks

INTRODUCTION

Although a large variety of halogenated hydrocarbons that occur in the environment are of natural origin, the widespread use and release of synthetic halogenated compounds in industry and agriculture over the past 100 years has introduced many man-made xenobiotic halogenated organics into the environment. Such synthetic organohalogens are generally more persistent than natural organohalogens. The lack of biodegradation is often due to the inability of microorganisms to effectively metabolize compounds with chemical structures to which microorganisms have not been exposed during the course of evolution. In other words, recalcitrance generally is the result of a lack of efficient metabolic pathways. Nevertheless, many examples are available of microorganisms that have the ability to metabolize xenobiotic organohalogens (see *Chapter 1* of this thesis). This led to the idea that such microorganisms must have evolved their catabolic pathways during the past few decades, and are thus suited to study the natural assembly of catabolic routes.

We decided that 1,3-dichloropropene (DCP) and 1,2-dibromoethane (DBE) are good model compounds to investigate the process of microbial adaptation to synthetic halogenated organic compounds for the following reasons. First, these compounds are not known to be produced naturally in significant concentrations (in contrast to many halogenated aromatics). Second, they were first introduced as nematocidic soil fumigants in the 1950s and are used in large quantities since that time. Third, no pure bacterial cultures were known that could use these xenobiotic pollutants as the sole carbon and energy source. Thus, our approach was to isolate bacterial strains that could aerobically utilize these xenobiotics as growth substrate and then characterize in detail the genetics and biochemistry of the corresponding pathways to establish the unique characteristics of DCP and DBE metabolism.

In the remainder of this Chapter we summarize our results and discuss the evolution of DCP and DBE degradative pathways, with the emphasis on the recruitment of the dehalogenating enzymes involved in the detoxification of these pollutants. Since it appeared that the gene encoding the first dehalogenating enzyme in both pathways originates from a haloalkane catabolic gene cluster that is widespread among gram-positive bacteria, we will first discuss the genetic organization of this globally distributed gene cluster.

GLOBAL DISTRIBUTION OF A GENE CLUSTER ENCODING HALOALKANE CATABOLISM

The capacity to use 1-chlorobutane and similar 1-halo-*n*-alkanes as a carbon source is widespread among gram-positive bacteria. Such organisms can easily be isolated from contaminated soil as well as from pristine soil or sludge samples (G.J. Poelarends, J.E.T. van Hylckama Vlieg, T. Bosma, and D.B. Janssen, unpublished observations). Several research groups have isolated and characterized gram-positive haloalkane degraders from widely separated geographical locations (Table 1). Some of these organisms were initially identified as strains of the genera *Arthrobacter*, *Corynebacterium* or *Acinetobacter* (12, 21, 33), but we showed that on the basis of 16S rRNA gene sequence analysis all these strains should be classified as strains of *Rhodococcus erythropolis* or a closely related species (*Chapter 5*).

Table 1. List of chloroalkane-degrading gram-positive strains carrying a highly conserved haloalkane catabolic gene cluster

Strain	Origin	Growth substrate	Reference
Strain Y2	UK	1-chlorobutane	20
Strain NCIMB13064	UK	1-chlorobutane	5
Strain m15-3	Japan	1-chlorobutane	32
Strain HA1	Switzerland	1-chlorohexane	21
Strain GJ70	Netherlands	1,6-dichlorohexane	12
Strain TB2	USA	1-chlorobutane	*Chapter 5*

Characterization of the 1-chlorobutane-utilizing bacterium *R. erythropolis* NCIMB 13064 led to a picture of the metabolic pathway for 1-chlorobutane degradation and of the different genes which are involved in this route (5, 15, 16, *Chapter 4*). The first step is the conversion of 1-chlorobutane to *n*-butanol by an inducible hydrolytic haloalkane dehalogenase (Fig. 1A). The alcohol intermediate is oxidized in two steps to *n*-butyric acid by the sequential action of an alcohol dehydrogenase (AdhA) and an aldehyde dehydrogenase (AldA). Butyric acid is further metabolized by β-oxidation to acetaldehyde, which then enters the central metabolic pathway. The different genes encoding these initial metabolic steps were found in a cluster on an autotransmissible 100 kb plasmid designated pRTL1 (Fig. 2) (*Chapter 4*). Directly upstream of the haloalkane-degradative genes, two additional genes, *dhaR* and *invA*, were found. The *dhaR* gene product is a member of the TetR family of transcriptional repressor-type regulators and appears to function as a repressor for the *dhaA* gene. The *invA* gene encodes a protein that shares extensive similarity with proteins belonging to the invertase family of site-specific recombinases. InvA is most similar to the well-characterized invertases

Pin of *Escherichia coli* (50%) and Hin of *Salmonella enterica* serovar Typhimurium (48%). The Pin and Hin invertases are responsible for the inversion of a specific DNA fragment that can serve as a genetic switch that determines the expression of alternative sets of genes (18, 35), but it is not known yet whether InvA plays an anologous role in regulating expression of haloalkane catabolic genes.

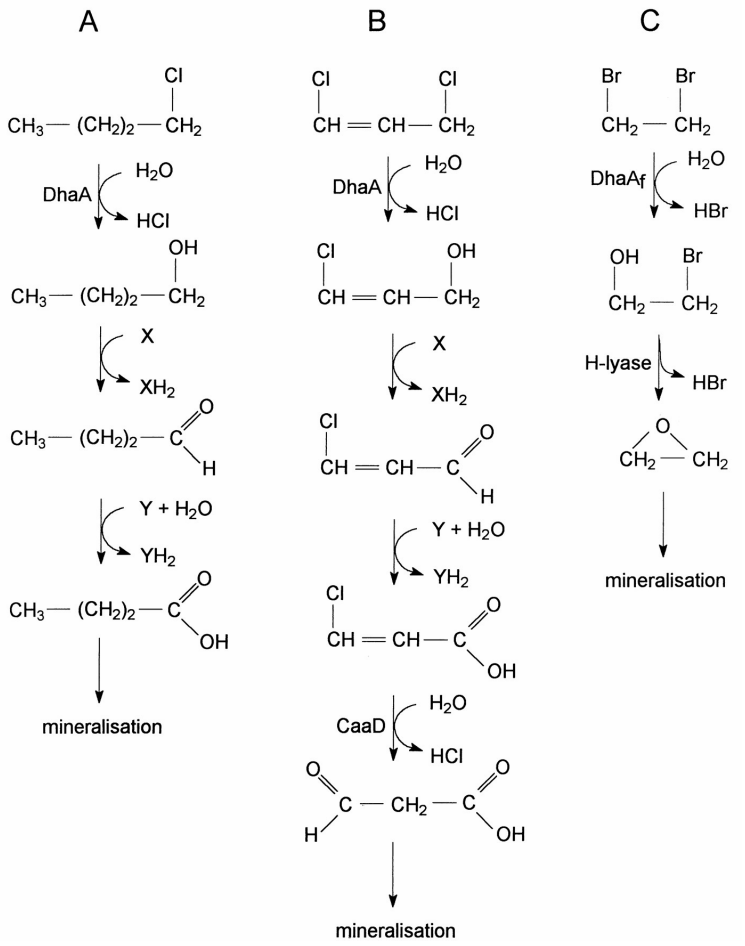

Figure 1. Initial enzymatic steps in the degradation of 1-chlorobutane by *R. erythropolis* NCIMB13064 (A), 1,3-dichloropropene by *P. pavonaceae* 170 (B), and 1,2-dibromoethane by *Mycobacterium* sp. strain GP1 (C). Abbreviations: DhaA and DhaA$_f$, haloalkane dehalogenases; AdhA, alcohol dehydrogenase; AldA, aldehyde dehydrogenase; HheB, haloalcohol dehalogenase; CaaD, *trans*-3-chloroacrylic acid dehalogenase; X, alcohol dehydrogenase cofactor; Y, aldehyde dehydrogenase cofactor.

A number of haloalkane dehalogenase-negative derivatives of strain NCIMB13064 were isolated. These spontaneous mutants showed rearrangements in both the chromosome and plasmid pRTL1 (15). Further analysis of the mutants revealed that a pRTL1-located insertion element, designated IS*2112*, was involved in these genome rearrangements, although no direct link between the rearrangements and IS*2112* transposition could be demonstrated

(14). Two copies of IS*2112* are present on pRTL1, but these sequences do not flank the haloalkane catabolic genes, and thus do not comprise a single composite transposon encoding haloalkane catabolism.

Until recently, nothing was known about the genes involved in haloalkane metabolism in the other five gram-positive strains listed in Table 1. The biochemical characteristics of the haloalkane dehalogenases isolated from these strains, however, closely resembled those of the haloalkane dehalogenase (DhaA) from *R. erythropolis* NCIMB13064 (6). This notion prompted us to test whether the haloalkane dehalogenase genes of these strains were similar to the *dhaA* gene of strain NCIMB13064. Remarkably, the haloalkane dehalogenase genes of these five haloalkane degraders, which were isolated from widely separated geographical locations, were all found to be completely identical to the *dhaA* gene (*Chapter 5*). Hybridization and sequence analysis of the genetic environment of the *dhaA* gene in each of the haloalkane degraders indicated high similarity to the haloalkane catabolic gene cluster found on plasmid pRTL1 in strain NCIMB13064 (Fig. 2) (*Chapters 4 and 5*). Apparently, the capacity to use haloalkanes as a carbon source has become widespread among gram-positive bacteria due to the global and recent distribution of a single catabolic gene cluster. In all strains analyzed, the gene cluster is localized on plasmids, suggesting a role for these mobile elements in gene transfer (*Chapter 5*).

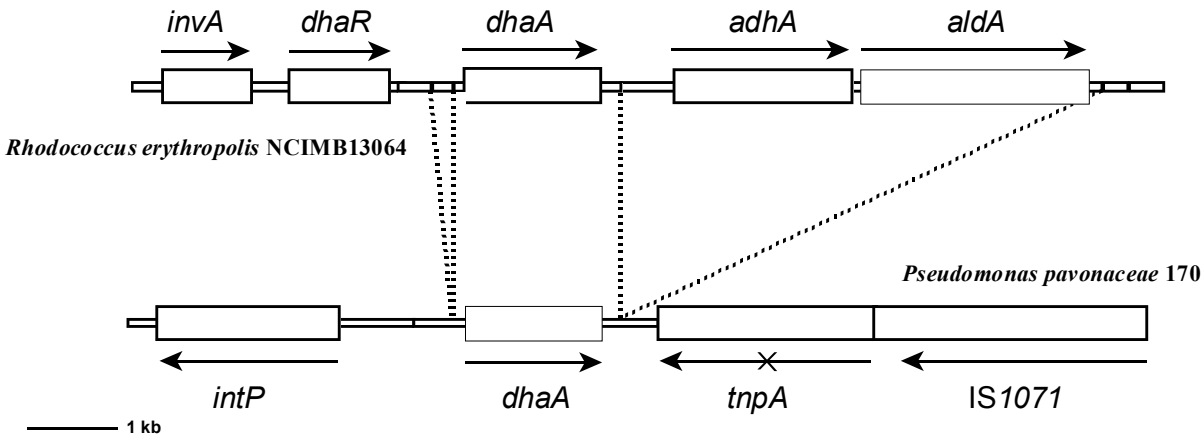

Figure 2. A comparison of the genetic environment of the haloalkane dehalogenase genes in *R. erythropolis* NCIMB13064 and *P. pavonaceae* 170. Genes are shown as boxes, and arrows indicate the direction of transcription. Identical hatching indicates identical sequences in both dehalogenase gene regions. The two deletions within the acquired genetic element in *P. pavonaceae* 170 are indicated by dotted lines. A putative transposase ORF (*tnpA*), which is located next to the acquired genetic element, is interrupted by an insertion element, identified as IS*1071* (*Chapter 4*). The *intP* gene encodes a putative (site-specific) DNA integrase.

EVOLUTION OF THE METABOLIC PATHWAY FOR DCP DEGRADATION: RECRUITMENT OF A HALOALKANE DEHALOGENASE THAT IS UNIQUE FOR GRAM-NEGATIVE BACTERIA

DCP is the major and active ingredient of the pesticidal formulations "D-D" (Shell Oil Company) and "Telone II" (Dow Chemical Company). These synthetic products are used worldwide in large amounts for the control of plant-parasitic nematodes. Fumigants such as D-D and Telone II represent an important class of carcinogenic water pollutants because their components can easily permeate through soils into groundwater supplies (4, 29). Initial studies indicated that the environmental degradation of both DCP isomers is mainly the result of microbial action, with the exception of the rate-limiting chemical hydrolysis of DCP to 3-chloroallyl alcohol (2, 19, 26). However, no definitive information was presented regarding the bacterial strains responsible for DCP degradation. The first report concerning enrichment and isolation of DCP-utilizing organisms was published in 1995 (30). In this report, Van Elsas and coworkers described that repeated treatment of soils with DCP resulted in accelerated biodegradation of this compound. Several DCP-degrading bacterial strains, belonging to the genera *Alcaligenes* and *Pseudomonas*, were isolated from such adapted soils. One strain was characterized and initially identified as *Pseudomonas cichorii* 170. On the basis of 16S rRNA gene sequence analysis, this organism was reclassified as *Pseudomonas pavonaceae* 170 (G. J. Poelarends and J. R. Marchesi, unpublished results).

Characterization of strain 170 (*Chapter 2*), along with 3-chloroallyl alcohol and 3-chloroacrylic acid-degrading strains isolated by others (1, 11, 27), led to a good picture of the metabolic pathway for DCP degradation (Fig. 1B). Although strain 170 was isolated solely for its ability to utilize *cis*-DCP as a growth substrate, it was also found capable of utilizing *trans*-DCP. In the first metabolic step, the DCP isomers are hydrolyzed to their corresponding 3-chloroallyl alcohol isomers by a haloalkane dehalogenase. Further conversion of the 3-chloroallyl alcohols proceeds via oxidation to *cis*- and *trans*-3-chloroacrylic acid. Both isomeric 3-chloroacrylic acid intermediates are converted to malonic acid semialdehyde, but by two different dehalogenating enzymes, one specific for *cis*-3-chloroacrylic acid and the other specific for *trans*-3-chloroacrylic acid. Malonic acid semialdehyde is further degraded via acetaldehyde, which enters the central metabolic pathways.

The haloalkane dehalogenase that catalyzes the first step in the degradation of DCP was purified to homogeneity (*Chapter 2*). Its biochemical characteristics and N-terminal amino acid sequence were indistinguishable from those of the haloalkane dehalogenase (DhaA) that was found thusfar only in gram-positive bacteria such as *R. erythropolis* NCIMB13064 (see above). PCR amplification of the haloalkane dehalogenase gene by using primers based on the expected sequence similarity to the *dhaA* gene, followed by DNA sequencing, revealed a sequence identical to that of *dhaA*. The presence and role of the *dhaA*

gene in the gram-negative strain 170 were confirmed by the isolation and characterization of haloalkane dehalogenase-negative mutants (*Chapter 2*).

From a detailed comparison of the genetic environment of the *dhaA* genes in *P. pavonaceae* 170 and *R. erythropolis* NCIMB13064 (Fig. 2), it appears that the *dhaA* gene of strain 170 originated from a haloalkane catabolic gene cluster that is widespread among gram-positive bacteria (*Chapter 4*). Most likely, a DNA segment containing the *dhaA* gene was horizontally transferred to strain 170. During or after this process, two deletions occurred within the recruited DNA segment (Fig. 2). One of these deletions includes the DNA sequence between a 13-bp directed repeat upstream of the *dhaA* gene, as well as one of the repeated sequences itself. The formation of this deletion may be explained by DNA strand slippage, which allows one repeated sequence to mispair with the complement of the other. The second deletion includes the genes for the alcohol dehydrogenase (*adhA*) and aldehyde dehydrogenase (*aldA*). Since *P. pavonaceae* 170 contains two other dehydrogenases that are involved in oxidation of 3-chloroallyl alcohol to 3-chloroacrylic acid, retention of the dehydrogenase genes in the acquired genetic element should not have been required.

From these analyses, it seems likely that horizontal transfer of a genetic element containing the *dhaA* gene from a gram-positive to a gram-negative bacterium played a role in the recent evolution of a gram-negative strain adapted to degrade DCP. The possibility that the 1-chlorobutane-catabolic gene cluster evolved in an (unknown) gram-negative species and subsequently became widely distributed among gram-positive bacteria, and was also transferred to *P. pavonaceae* 170, must be considered as well. DCP is lethal to a strain of *P. pavonaceae* 170 which has lost the haloalkane dehalogenase gene (G. J. Poelarends and D. B. Janssen, unpublished results). Thus, only the complete pathway allows *P. pavonaceae* 170 to grow in the presence of DCP.

CONSIDERATIONS OF THE EVOLUTIONARY ORIGIN OF *TRANS*-3-CHLOROACRYLIC ACID DEHALOGENASE

Trans-3-chloroacrylic acid dehalogenase (CaaD) is the second dehalogenating enzyme in the DCP degradation pathway in *P. pavonaceae* 170 (Fig. 1B). It catalyzes the hydrolytic dehalogenation of *trans*-3-chloroacrylic acid to yield malonic acid semialdehyde. Mature CaaD was found to be a hexamerix protein of 50 kDa that consists of three α-subunits of 75 amino acid residues and three β-subunits of 70 residues (CaaD is postulated to be a trimer of αβ-dimers) (*Chapter 6*). A homologue of CaaD has been found in the 3-chloroacrylic acid-degrading coryneform bacterial strain FG41 (27). So far, no natural substrate for CaaD has been identified, and hydrolytic dehalogenation of *trans*-3-chloroacrylic acid and *trans*-3-bromoacrylic acid is the only known activity for the enzyme. The precursor of *trans*-3-

chloroacrylic acid in *P. pavonaceae* 170, *trans*-DCP, is not known to be formed naturally. The industrial production of *trans*-DCP started in the 1950s (29). Therefore it is reasonable to propose that CaaD arose from an existing enzyme, catalyzing the conversion of some other substrate in soil bacteria, with a structural scaffold and catalytic groups that allow adaptation to *trans*-3-chloroacrylic acid. It is conceivable that such an enzyme happened to have some level of dehalogenase activity in the initial encounter with *trans*-3-chloroacrylic acid.

In an attempt to find the origin of CaaD by homology analysis, we discovered that CaaD has low but significant sequence identity to 4-oxalocrotonate tautomerases (4-OTs) (*Chapter 6*). 4-OTs catalyze the isomerization of 2-oxo-4-hexene-1,6-dioate to 2-oxo-3-hexene-1,6-dioate during the catabolism of catechol to metabolites in the Krebs cycle (3, 24). They are also hexameric proteins consisting of small subunits (62 to 79 amino acid residues), but, in contrast to CaaD, they are composed of identical subunits (4-OT is a trimer of homodimers). Certain aspects of the proposed mechanism of the CaaD-catalyzed reaction (*Chapter 6*) bear a strong resemblance to the mechanism of 4-OTs (Fig. 3). Both 4-OT and CaaD utilize their amino-terminal proline (Pro-1) as proton acceptor/donor, and in both proteins Arg-11 plays an important role in substrate binding. Consistent with the location of Pro-1 and Arg-11 on adjacent subunits within the same homodimer of the 4-OT molecule, the catalytically important proline of CaaD is located on the β-subunit, whereas Arg-11 is located on the α-subunit, showing that residues from both subunits contribute to the dehalogenase active site. The role of these active site residues in CaaD has been validated by site-directed mutagenesis; the substitutions βP1A/G and αR11A resulted in inactive enzymes, whereas the substitution αP1A/G had no significant influence on dehalogenase activity (*Chapter 6*). Although CaaD has a low overall identity to the known 4-OTs, the active site region around Arg-11 is highly conserved (Fig. 4A).

On the basis of these structural and mechanistic similarities, we suggest that CaaD shares a common ancestry with 4-OTs. However, CaaD does not share high sequence identity to the known 4-OTs, indicating that the time of branching from a common ancestor occurred long ago. Thus, the recent relative(s) from which CaaD evolved in response to the introduction of DCP into the environment is not known. It should be noted, however, that prior to the introduction of *trans*-DCP into the environment by human activities, *trans*-3-chloroacrylic acid (or a structurally related compound) might have been formed from a natural product, for example a chlorinated aromatic compound. Consequently, the possibility that CaaD arose from a dehalogenase that is involved in the conversion of such a naturally produced organohalogen should be considered as well.

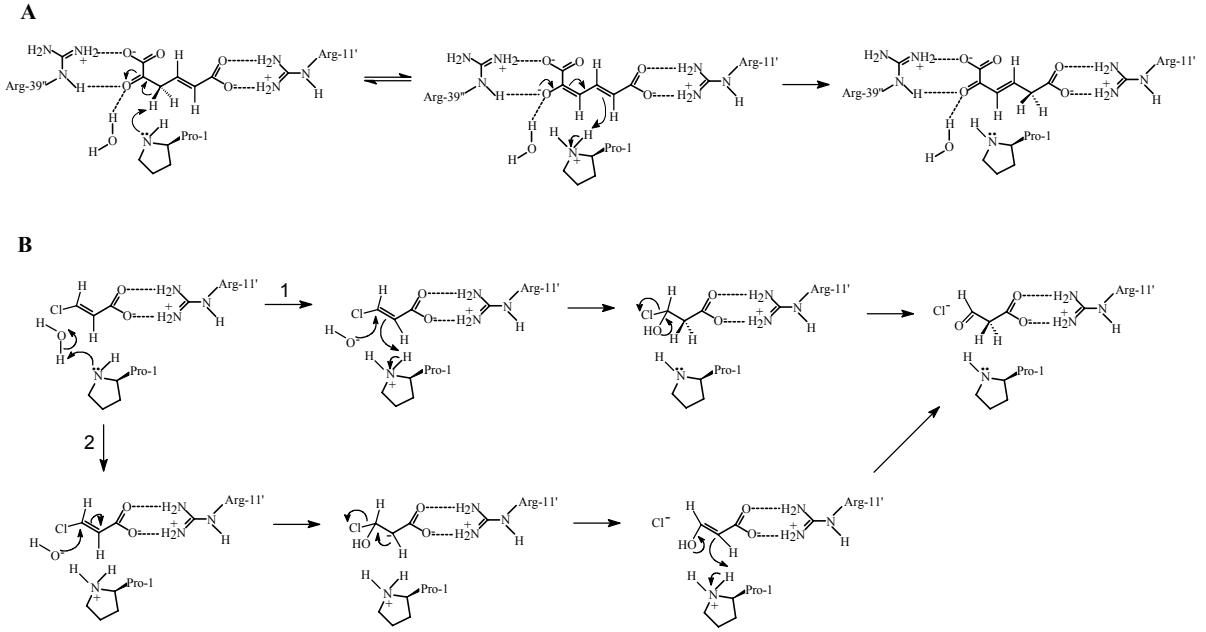

Figure 3. A comparative reaction scheme for 4-oxalocrotonate tautomerases and *trans*-3-chloroacrylic acid dehalogenase. Primed residues come from other subunits. (A) The reaction catalyzed by 4-OTs (modified from reference 10). (B) The reaction catalyzed by CaaD. The order of protonation at C-2 and Cl^- departure is uncertain, so two possible reaction schemes are shown: one involving the formation of the intermediate 3-chloro-3-hydroxypropanoic acid (route 1) and the other involving the formation of a carbanion intermediate (route 2).

EVOLUTION OF THE METABOLIC PATHWAY FOR DBE DEGRADATION: A NOVEL COMBINATION OF TWO EXISTING DEHALOGENASE GENES

DBE is an effective nematocidic agent of which more than 20 million pounds were used for soil fumigation in the United States before it was banned in 1983 by the U.S. Environmental Protection Agency because of its carcinogenic properties and widespread occurrence in groundwater supplies (25). Many years after its last application, residual DBE can still be found in soil because it strongly interacts with the soil matrix and it is highly resistant to microbial degradation (22, 31). DBE can slowly leach from such contaminated soils to groundwater over extremely long periods, and due to its slow chemical conversion in aqueous milieu, it forms a continuing threat for groundwater quality (22).

Many attempts to obtain pure cultures of bacteria that can degrade DBE have been unsuccessful, although the structural analog 1,2-dichloroethane (DCE) can be utilized by various bacteria of the genera *Xanthobacter* and *Ancylobacter* (13). These bacteria degrade DCE by hydrolytic conversion to 2-chloroethanol, which is oxidized via chloroacetaldehyde to chloroacetate and finally hydrolyzed to glycolic acid. DCE-degrading bacteria thus seem to

A

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CaaD1       PMISCDMRYGRTDEQKRALSAGLLRVISEATGEPRENIFFVIREGSGINFVEHGEHLPDYVPGNANDKALIAKLK---- 75
4-OT-CF600  PIAQLYIIEGRTDEQKETLIRQVSEAMANSLDAPLERVRVLITEMPKNHFGIGGEPASKVR--------R--------- 62
4-OT-mt-2   PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKVR--------R--------- 62
            *        ** **** *              *       * *     *   **
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B

```
CaaD2       PFIECHIATGLSVARKQQLIRDVIDVTNKSIGSDPKIINVLLVEHAEANMSISGRIHGEAASTERTPAVS--------- 70
4-OT-CF600  PIAQLYIIEGRTDEQKETLIRQVSEAMANSLDAPLERVRVLITEMPKNHFGIGGEPASKVRR---------------- 62
4-OT-mt-2   PIAQIHILEGRSDEQKETLIREVSEAISRSLDAPLTSVRVIITEMAKGHFGIGGELASKVRR---------------- 62
            *     *  *     *  *** *      *         *   *       * *
```

Figure 4. Amino acid sequence alignment of the α-subunit (CaaD1) or β-subunit (CaaD2) of CaaD with two well-characterized 4-OTs. The catalytically essential residues in CaaD and 4-OTs are shown in boldface. The region of highest sequence identity among CaaD1 and the 4-OTs is boxed. 4-OT-mt-2, 4-oxalocrotonate tautomerase from *Pseudomonas putida* mt-2 (accession no. Q01468); 4-OT-CF600, 4-oxalocrotonate tautomerase from *Pseudomonas* sp. CF600 (accession no. P49172).

possess all the enzymes necessary to degrade DBE, but they are not able to utilize DBE because both DBE and the expected intermediate 2-bromoethanol are toxic at concentrations in the μM range. The presence of an active alcohol dehydrogenase in the absence of a functional aldehyde dehydrogenase, which causes the accumulation of the highly reactive bromoacetaldehyde, seems to be the cause of this toxicity (28).

The first report concerning microbial utilization of 1,2-dibromoethane as a carbon source was published in 1996 (8). In this report, Freitas dos Santos and coworkers described the enrichment of a mixed bacterial culture capable of slow aerobic mineralization of DBE. We succeeded in isolating from this consortium a pure bacterial culture (*Mycobacterium* sp. strain GP1) that can aerobically utilize DBE as the sole carbon and energy source (*Chapter 3*). It was obtained by prolonged adaptation and selection in batch culture. Metabolism of DBE by strain GP1 was initially expected to proceed via a modified DCE-degradation route, in which the aldehyde dehydrogenase should be overexpressed or adapted so that no accumulation of the toxic intermediate bromoacetaldehyde would occur. Surprisingly, strain GP1 utilizes another pathway in which DBE is metabolized via the sequential action of a hydrolytic haloalkane dehalogenase and a haloalcohol dehalogenase (Fig. 1C). The latter enzyme was found to be highly active towards 2-bromoethanol and rapidly converted this intermediate to ethylene oxide. In this way, the organism circumvents the formation of the toxic intermediate bromoacetaldehyde, which was lethal when DCE-degrading bacteria were exposed to DBE. Although ethylene oxide is toxic as well, pathways for its degradation have been described (7). Complete metabolism of DBE thus employs two dehalogenating enzymes to prevent the formation of toxic brominated intermediates.

The genomic region of strain GP1 that encodes the dehalogenases has been cloned and sequenced (Fig. 5) (*Chapters 3 and 4*; Van Hylckama Vlieg et al., unpublished data).

Information that might indicate the genetic origin of the haloalkane dehalogenase (*dhaA$_f$*) and haloalcohol dehalogenase (*hheB*) genes comes from a comparison with the sequence of the dehalogenase gene regions of *R. erythropolis* NCIMB13064 (see above) and *Corynebacterium* sp. strain N-1074 (34). Strain GP1 harbors a genetic element, containing *dhaR*, *dhaA*, and part of *invA*, that is highly similar to a segment of the 1-chlorobutane catabolic gene cluster found in gram-positive bacteria such as strain NCIMB13064 (Fig. 2). The *hheB* gene and its flanking regions in strain GP1 are highly similar to the *hheB* gene region of the haloalcohol-degrading strain N-1074. From these findings, it appears that in strain GP1 two existing genetic regions for dehalogenation are combined, one containing the *dhaA* gene (segment A in Fig. 5) and the other containing the *hheB* gene (segment B in Fig. 5). The combination of these two dehalogenase genes in a single organism resulted in a productive pathway for DBE degradation. Since strain GP1 was isolated by prolonged adaptation of a mixed batch enrichment culture (*Chapter 3*), it is likely that recent genetic recombination events led to the formation of this novel dehalogenase gene cluster.

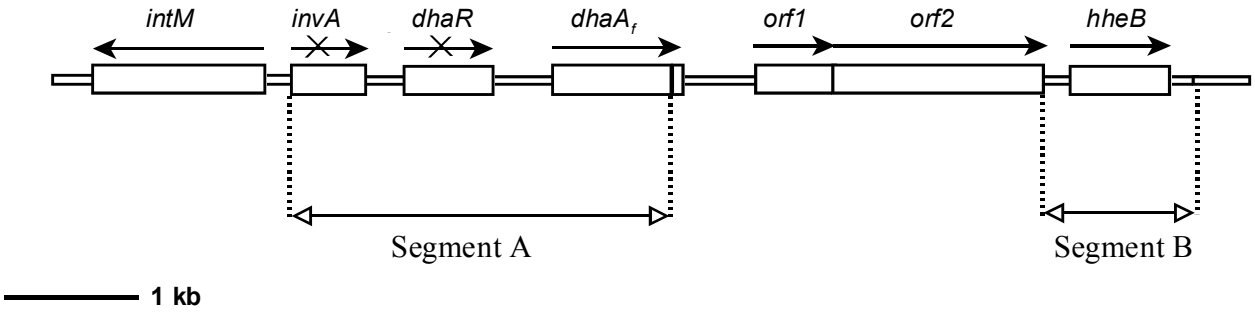

Figure 5. Genomic region of *Mycobacterium* sp. strain GP1 encoding the haloalkane (DhaA$_f$) and haloalcohol (HheB) dehalogenases. Segment A is identical to a segment of the haloalkane catabolic gene cluster found in gram-positive bacteria such as *R. erythropolis* NCIMB13064, except for three nucleotide substitutions in *dhaA* and a 12-nucleotide deletion in *dhaR*. Segment B is identical to a segment of the haloalcohol dehalogenase gene region in *Corynebacterium* sp. strain N-1074, except for four nucleotide substitutions in *hheB*. The 42-nucleotide extension of the *dhaA* ORF, which is the result of a fusion between the acquired genetic element and HheB encoding DNA, is indicated by an open box. The function of the putative ORF1 and ORF2 proteins is not known. The *intM* gene encodes a putative (site-specific) DNA integrase.

At least two other genetic alterations appear to have occurred in the dehalogenase region of the GP1 genome. First, we found that the *dhaA* gene is fused in frame to a segment of the *hheB* gene, resulting in an extension of the *dhaA* open reading frame (ORF) with 42 nucleotides (i.e. 14 amino acids at the protein level) (Fig. 5). This new ORF was accordingly named *dhaA$_f$* (were f stands for fusion). Since this *hheB* part of *dhaA$_f$* is identical to the 3' end

of the intact *hheB* gene that is located approximately 2.6 kb downstream of $dhaA_f$ (see Fig. 5), it seems likely that a duplication event leading to two copies of part or the whole *hheB* gene occurred prior to acquisition and insertion of the *dhaA* gene. Nevertheless, it is surprising that the recruited *dhaA* gene became fused to the *hheB* gene, which normally codes for a haloalcohol dehalogenase that could be involved in the metabolism of DBE. The 14-amino-acid extension has no influence on the activity of DhaA, however, and may just reflect the recombination events that led to acquisition of a catabolic pathway. Second, a deletion of 12 nucleotides has occurred within the *dhaR* gene, which normally encodes a repressor (DhaR) for the *dhaA* gene. Although this deletion is in frame, it inactivated the DhaR protein, leading to constitutive expression of $dhaA_f$ (*Chapter 4*). It may have been a necessity for strain GP1 to inactivate this protein since the compound used for selection (DBE) is known not to be an inducer of *dhaA* expression.

DO DNA INTEGRASES PLAY A ROLE IN THE ACQUISITION OF DEHALOGENASE GENES?

Acquisition of foreign DNA by horizontal gene transfer requires integration into a replicon that is stable maintained in the recipient microorganism. Interestingly, both in *P. pavonaceae* 170 and in *Mycobacterium* sp. strain GP1 a gene encoding a putative site-specific recombinase (*intP* in strain 170 and *intM* in strain GP1) is present directly upstream of the recruited DNA segment harboring the haloalkane dehalogenase gene (Fig. 2 and 5). The *intP* and *intM* gene products share significant sequence similarity with members of the integrase (Int) family of site-specific recombinases, and both harbor the conserved, catalytically important, tetrad R-H-R-Y of the Int family (*Chapter 4*). These putative integrase proteins probably mediated the insertion of the dehalogenase genes into the genome, although their activity remains to be established experimentally. The finding of putative integrase genes next to the dehalogenase genes further reinforces our hypothesis that these DNA segments were acquired by horizontal transmission.

Integrase-mediated gene acquistion has previously been associated with an unique class of genetic elements, called integrons (23). Integrons harbor a gene for a site-specific DNA integrase, which can mediate the incorporation of one or more foreign genes in a specific site (the recombination or core site) directly upstream of the integrase gene. Thusfar, integrons have been implicated in the acquisition of antibiotic resistance genes by various bacterial species (23) and of virulence determinants by *Vibrio cholerae* (17). Integrons harboring antibiotic resistance genes may be mobilized after association with insertion elements, transposons, or conjugative plasmids. This fascinating class of genetic elements thus forms an extremely powerful mechanism for the acquisition and distribution of genes involved in adaptation or colonization of new environments.

The acquired DNA segments in *P. pavonaceae* 170 and *Mycobacterium* sp. strain GP1 lack the specific structural characteristics of previously identified integron-inserted gene cassettes, which consist of an imperfect inverted repeat located at the 3'end of the inserted gene (called a 59-base element) and two core sites (GTTRRRY) located at the boundaries of each inserted gene cassette (9). Nevertheless, the localization of an integrase gene directly next to an apparently recently assembled genetic element suggests an integron-like structure. The lack of high sequence similarity of IntP and IntM with known integron encoded DNA integrases may explain why the structural features of the inserted gene cassettes are not conserved in strains 170 and GP1. Thus, it will be interesting to find out whether dehalogenase genes can be propagated via integron-like elements.

CONCLUSIONS AND FUTURE DIRECTIONS

The isolation of pure bacterial cultures capable of utilizing and degrading the priority pollutants DCP and DBE is important for understanding their environmental fate and makes it possible to develop bioreactors for the removal of these compounds from contaminated groundwater. Moreover, these organisms provide nice examples of the different adaptation mechanisms that are involved in the generation of new catabolic pathways, i.e. horizontal transmission of degradative genes, insertion of these genes into the genome by means of an integration system, inactivation of a repressor of a catabolic gene, and the circumvention of the formation of toxic catabolic intermediates.

The data presented in this thesis clearly show that microorganisms have assembled new metabolic pathways by the recruitment and novel association of existing dehalogenating enzymes. Questions concerning the evolutionary origin of these enzymes (and of dehalogenases in general) and the adaptive mutations that may have occurred in the initial encounter with xenobiotic organohalogens remain to be answered. This requires insight into all potential sources of DNA encoding (ancestral forms of) dehalogenases in the environment. We envisage the following sources: (i) Organohalogen-producing organisms. Such organisms may synthesize dehalogenating enzymes to protect themselves against the toxic effects of their metabolites. (ii) Organohalogen-degrading organisms. Organisms degrading naturally produced organohalogens may possess the ancestral forms of the dehalogenases involved in the degradation of synthetic organohalogens. Candidates are organisms degrading abundant natural organohalogens such as haloaromatics and their degradation products. (iii) Organisms-degrading non-halogenated structural analogs of synthetic organohalogens. Such organisms may possess enzymes that were recruited to serve as dehalogenases in the biodegradation of xenobiotics. Insight into the evolutionary origins of dehalogenases involved in the conversion

of xenobiotics thus awaits the isolation and characterization of dehalogenase genes from these potential sources.

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Samenvatting

Van verschillende gehalogeneerde alifatische koolwaterstoffen wordt aangenomen dat ze pas in de biosfeer voorkomen sedert ze industrieel gesynthetiseerd worden. Dit verklaart het recalcitrante gedrag van deze verbindingen: er zijn nauwelijks micro-organismen die ze als groeisubstraat kunnen gebruiken. Voor een aantal stoffen, waaronder 1,2-dichloorpropaan en 1,2,3-trichloorpropaan, is het nooit gelukt organismen te vinden die erop groeien. Afbraak is dan uiterst langzaam en afhankelijk van cometabolisme. Oorzaken voor de slechte afbreekbaarheid van gehalogeneerde koolwaterstoffen zijn in het algemeen het ontbreken van microbiële enzymen (dehalogenasen) die deze synthetische milieuvreemde stoffen als substraat kunnen gebruiken en de giftigheid van deze verbindingen of de gevormde tussenproducten na omzetting. Biochemische factoren zijn dus de oorzaak van de slechte afbreekbaarheid, want gehalogeneerde alifatische koolwaterstoffen leveren ruim voldoende energie voor aërobe groei.

Voor andere stoffen kunnen organismen die tot afbraak in staat zijn soms wel geïsoleerd worden uit entmateriaal van plaatsen die al langere tijd met dergelijke stoffen zijn gecontamineerd. Deze organismen moeten dus beschikken over enzymen die milieuvreemde structuren (ook wel xenobiotica genoemd) herkennen en omzetten. Over de evolutionaire oorsprong van deze enzymen en de mechanismen van adaptatie is slechts weinig informatie beschikbaar. Het onderzoek heeft zich daarom gericht op het verkrijgen van inzicht in de manier waarop xenobiotica-afbrekende activiteiten zich ontwikkelen en verspreiden. De synthetische chemicaliën 1,3-dichloorpropeen en 1,2-dibroomethaan werden gekozen als model verbindingen voor het bestuderen van microbiële adaptatie. Deze stoffen worden sinds 1950 op grote schaal toegepast als grondontsmettingsmiddelen (nematociden) in de akkerbouw, de boomkwekerij en de bloemen- en plantenteelt. Ze zijn vreemd voor de natuur en worden niet of slecht afgebroken door micro-organismen. Ten gevolge van de slechte biologische afbraak kunnen deze mutagene en kankerverwekkende stoffen accumuleren in het grondwater, waar zij een sterke bedreiging vormen voor de kwaliteit van het drinkwater en de daar aanwezige micro-organismen. De experimentele aanpak betrof dus de isolatie van micro-organismen die 1,3-dichloorpropeen of 1,2-dibroomethaan als groeisubstraat konden gebruiken, om vervolgens in detail de genetica en biochemie van de corresponderende afbraakroutes te bestuderen en te vergelijken met katabole routes en genen in andere (xenobiotica-afbrekende) micro-organismen.

Het bleek inderdaad mogelijk om twee bacteriële stammen te isoleren die in staat zijn om 1,3-dichloorpropeen of 1,2-dibroomethaan als enige koolstof- en energiebron te gebruiken. De 1,3-dichloorpropeen-afbrekende bacterie *Pseudomonas pavonaceae* 170 werd geïsoleerd uit entmateriaal verkregen van landbouwgrond die meerdere malen behandeld was met 1,3-dichloorpropeen. Stam 170 zet deze verbinding om via chloorallylalcohol en

chlooracrylzuur tot acetaldehyde. Dit organisme maakt gebruik van drie dehalogenasen om op beide isomeren van 1,3-dichloorpropeen te kunnen groeien: een haloalkaan dehalogenase, dat beide isomeren van 1,3-dichloorpropeen omzet in de corresponderende chloorallylalcoholen, en twee chlooracrylzuur dehalogenasen, één specifiek voor het *trans*-isomeer, de ander specifiek voor het *cis*-isomeer van chlooracrylzuur. Stam 170 beschikt dus over dehalogenerende enzymen die de milieuvreemde en giftige verbindingen *cis*- en *trans*-1,3-dichloorpropeen herkennen en omzetten tot onschadelijke producten.

Het meest bijzonder zijn de chlooracrylzuur dehalogenasen. Deze enzymen zijn in staat een chloor af te splitsen van een vinylisch koolstofatoom zonder dat eerst activatie tot een coenzym A derivaat nodig is. Er werd daarom besloten om het reactie-mechanisme van deze enzymen nader te bestuderen. Daartoe werden de genen die coderen voor het *trans*-3-chlooracrylzuur dehalogenase gekloneerd en werd het tot overexpressie gebrachte enzym bestudeerd. Het dehalogenase herkent slechts twee substraten (*trans*-3-chloor- en *trans*-3-broomacrylzuur) en is een hexameer bestaande uit opmerkelijk kleine subunits van 7.6 en 8.4 kDa. De aminozuur-sequentie van het dehalogenase vertoont significante identiteit met 4-oxaalcrotonaat tautomerases. Deze tautomerases zijn ook hexamere enzymen, echter zij bestaan uit identieke subunits van 62 aminozuren. Van enkele tautomerases is de driedimensionale structuur en het reactie-mechanisme bekend. Op basis van de sequentie homologie met 4-oxaalcrotonaat tautomerases en plaats-specifieke mutatie experimenten zijn de katalytisch belangrijke residuen in *trans*-3-chlooracrylzuur dehalogenase geïdentificeerd, wat leidt tot een reactie-mechanisme. De amino-terminale proline in de β-subunit fungeert waarschijnlijk als base en activeert een watermolecuul, dat vervolgens een aanval uitvoert op het koolstofatoom waaraan de chloor of broom gebonden is. Dit resulteert in een onstabiel tussenproduct dat uiteenvalt in malonaat semialdehyde en halide. Een arginine residue (Arg-11 in de α-subunit) is waarschijnlijk belangrijk voor de binding en positionering van het substraat in het actieve centrum. Op basis van de overeenkomsten in structuur en mechanisme, wordt geconcludeerd dat 4-oxaalcrotonaat tautomerases en 3-chlooracrylzuur dehalogenases evolutionair verwant zijn, ze bezitten een gemeenschappelijke "voorouder".

De 1,2-dibroomethaan-afbrekende bacterie *Mycobacterium* sp. stam GP1 werd geïsoleerd vanuit een 1,2-dibroomethaan-afbrekende microbiële mengculture door middel van langdurige selectie-experimenten. Deze stam heeft waarschijnlijk zijn afbrekende eigenschappen verkregen tijdens deze adaptatie-periode. Stam GP1 gebruikt een bijzondere katabole route die bestaat uit twee opeenvolgende dehalogenatie stappen om 1,2-dibroomethaan te ontgiftigen tot ethyleen oxide, dat vervolgens als koolstofbron wordt gebruikt. Een haloalkaan dehalogenase zet 1,2-dibroomethaan om in 2-broomethanol, dat vervolgens zeer snel wordt omgezet in ethyleen oxide door een haloalcohol dehalogenase. De snelle omzetting van 2-broomethanol is de belangrijkste stap in de afbraak van 1,2-dibroomethaan, omdat op deze manier de vorming van het giftige tussenproduct broomacetaldehyde wordt voorkomen. Het haloalcohol dehalogenase gen werd recent

gekloneerd, waardoor het nu mogelijk is om het reactie-mechanisme van het enzym nader te bestuderen.

In zowel *Pseudomonas pavonaceae* 170 als *Mycobacterium* sp. stam GP1 wordt de afbraak van haloalkanen dus geïnitieerd door een haloalkaan dehalogenase. De sequentie en genetische organisatie van de corresponderende haloalkaan dehalogenase genen werd bepaald en vergeleken met katabole genen in verschillende haloalkaan-afbrekende micro-organismen om inzicht te verkrijgen in de oorsprong van deze genen. Dit vergelijkend onderzoek heeft aangetoond dat stammen 170 en GP1 de haloalkaan dehalogenase genen verkregen hebben via horizontale gen overdracht, waarna de genen vermoedelijk geïnserteerd zijn in het genoom met behulp van DNA integratie-systemen. Beide genen zijn waarschijnlijk afkomstig van een katabole gencluster die aanwezig is in vele haloalkaan-afbrekende *Rhodococcus erythropolis* stammen geïsoleerd uit gecontamineerde en niet-gecontamineerde grond monsters. Horizontale overdracht van bestaande dehalogenase genen speelt dus een belangrijke rol in de adaptatie aan gehalogeneerde koolwaterstoffen. Het vergelijkend onderzoek heeft bovendien aanwijzigingen gegeven dat deleties en transpositie een belangrijke rol spelen in genetische adaptatie.

Samenvattend, de resultaten beschreven in dit proefschrift geven aan dat de katabole routes voor de afbraak van de milieuvreemde stoffen 1,3-dichloorpropeen in *Pseudomonas pavonaceae* 170 en 1,2-dibroomethaan in *Mycobacterium* sp. stam GP1 recentelijk (binnen 50 jaar) gevormd zijn door de acquisitie en unieke combinatie van bestaande dehalogenerende enzymen. Deze recentelijk geëvolueerde afbraak routes zijn op genetisch niveau geassocieerd met integrase genen, suggererend dat DNA integrases een belangrijke rol spelen in de acquisitie van katabole genen. Hoogst interessant is het voorkomen van haloalkaan dehalogenase genen in de genoom sequenties van micro-organismen die niet betrokken zijn bij de afbraak van gehalogeneerde xenobiotica. Dit suggereert dat haloalkaan dehalogenasen oude enzymen zijn. De fysiologische rol en natuurlijke substraten van deze enzymen zijn nog onbekend.

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